

nature

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Windscale issues need more debate

It is now two weeks since the publication of Mr Justice Parker's report, *The Windscale Inquiry*—the result of a hundred days of investigation into the application of British Nuclear Fuels Ltd (BNFL) for planning permission for 'a plant for reprocessing irradiated oxide nuclear fuels and support site services'. Two weeks in which the wide range of objectors have had a chance to absorb the criticism levelled at many of them and to start issuing responses. Two weeks in which Members of Parliament, to whom the decision now passes, have presumably been doing their homework.

Mr Peter Shore, Secretary of State for the Environment, in a parliamentary manoeuvre, rejected the application (favourable as Mr Parker's report was) in order to allow debate in public and in the House before a decision was taken.

There is no doubt that the document as a whole is impressive; no-one could be left with the impression that Mr Parker had not hoisted in all the technical questions. Nor on the whole can it be claimed that he was insensitive to the broad issues of principle which divided BNFL and the objectors. On the other hand, it does somehow seem as if once Mr Parker had decided that the decision should go in BNFL's favour he went out of his way to find for them on almost every issue—rather as a judge, confronted by a bunch of witnesses prepared to testify to a man's innocence, might dismiss their evidence *in toto* once satisfied that the man was guilty.

Should Britain go ahead with an oxide reprocessing plant to cope with the arisings from Advanced Gas-Cooled Reactors, possible Light-Water Reactors and foreign sources, in the first instance from Japan? We believe, unlike Mr Parker, that the case is not strong enough at present, and that there are good reasons for delaying for a few years.

The central plank in the argument in favour of going ahead now is that energy forecasts point to the necessity of bringing power reactors on stream regularly in the coming decades. Mr Parker was clearly exasperated by some of the details he heard about energy futures, for he speaks of some evidence as notably lacking in moderation and rational argument. He also notes that some parties seemed completely to overlook the great difference between making a forecast and having to take a decision which will affect the lives of millions. This seems to be offered as justification for taking only the government's forecasts into account except where objectors' evidence can be used in support of BNFL. In this way it is easy to see the desirability of building AGRs, but is not possible that within the wide spectrum of energy futures produced by people outside government there might be something every bit as convincing, not calling for such wholehearted nuclear commit-

ment? Certainly the inquiry was given evidence, not mentioned in the report, of such futures which even had growth built into them. It may well have been a misapprehension of Mr Parker, fuelled by an almost religious zeal in some quarters, that non-nuclear futures mean a decline in living standards. The business of energy forecasting is developing so rapidly and is turning up such interesting results that for this reason alone delay could be justified simply in order to learn more about what other options might be open to us.

The other major issue on which we believe the report can be criticised is that of proliferation. Indisputably there is more than one side to this question; for instance, reprocessing in Britain could be seen as a way of diminishing the need for other countries to set up their own plants, or it could be seen as a way of providing some sort of legitimisation for them to do so themselves. Mr Parker scrutinised the Non-Proliferation Treaty and came up (see page 302) with the opinion that the treaty, if anything, positively encouraged the establishment of a reprocessing facility for handling foreign fuel. This opinion may be legally correct in the sense that it is what parties to the treaty would have held in 1970, but a strict legal construction of an admittedly imperfect document eight years on seems wholly out of place. What is important is what people feel now about proliferation (though it must be admitted that the British government has done little to stimulate thinking on this matter outside official circles). If the Parker recommendation stands, certainly we will be able to offer foreign countries a reprocessing service, but no country will be obliged to use it. Any country looking to acquire nuclear weapons can simply respond that our price is wrong, or that its own nuclear engineers need employment building a reprocessing plant. And on legitimisation we have a recent example to bear in mind: the Indian nuclear explosion. In 1974 the Indians detonated their first nuclear device. Since the Americans and Russians had spent much of the preceding decade extolling the virtues of peaceful nuclear explosions the Indians called their device peaceful too, to considerable diplomatic confusion.

We do not know that Windscale will help, physically or morally, any other country to get into the weapons business. It may very well not. But it ought to be clear that discussion on proliferation issues is as yet too little advanced for such a major step to be taken.

A planning inquiry, however broad ranging, is not the place for the weighty matters of energy forecasting and nuclear proliferation to be resolved. These belong in the political arena and should not be decided in haste. □





'A complete vindication of BNFL's proposal . . . I am confident that the debate will confirm this' Con Allday, Managing Director of British Nuclear Fuels Ltd

The thermal oxide reprocessing plant (THORP) which British Nuclear Fuels Limited (BNFL) has plans to build at Windscale in the north west of England will cost an estimated £600 million and will take about ten years to build. If the UK Parliament gives BNFL the go ahead, the new plant should be ready to start reprocessing British and foreign oxide fuel by the early 1990s. Its total annual reprocessing capacity will be about 1,200 tonnes but initially at least, it is likely that it will only operate at about half its capacity.

Reprocessing spent uranium-metal fuel from Britain's Magnox reactors has been going on at Windscale since 1951. The oxide fuel which THORP is designed to reprocess, however, is used in light water reactors and advanced gas cooled reactors (AGRs). These are widely used throughout the world and will make up the next generation of British reactors.

BNFL's plan for THORP is for a complete reprocessing complex. It includes receipt and storage facilities for incoming fuel and facilities for chopping, dissolution, chemical separation, conversion of the purified uranium and plutonium to oxides, and treating storing and disposing of solid, liquid and gaseous waste. The storage pond facilities for incoming fuel are designed to be adequate to store the plant's maximum fuel capacity for at least a year.

According to the plan, irradiated oxide fuel going to THORP for reprocessing will first be stored in water filled bottles in deep water ponds to allow short lived fission products to decay. After at least one year's storage, the fuel will be taken to the 'head end plant' where it will be sheared into 2 inch pieces and dissolved in nitric acid. The acid solution will enter the first solvent extraction process where the bulk of the fission products will be removed in 'pulsed columns'. Subsequent

The Windscale Report

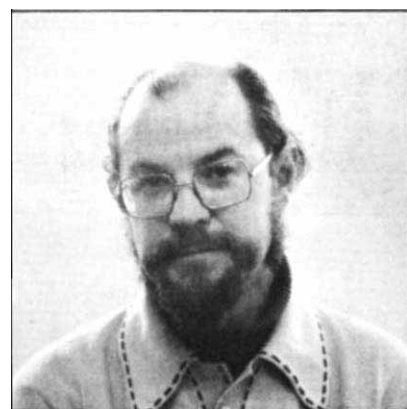
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solvent extract in mixer settlers will separate plutonium nitrate solution and uranyl nitrate, both of which will be further treated to form plutonium oxide and uranium oxide powders. These will either be stored or reused in new fuel.

The highly active waste from the first extraction cycle will be transferred to an evaporation plant for concentration and storage. It will be stored in double-walled, water cooled tanks until it can be vitrified. Medium and low active wastes will also be concentrated and stored to allow some decay before being sampled and released to the environment.

BNFL already has some experience of reprocessing oxide fuel. In 1969 it added a pretreatment section (B204) to its Magnox reprocessing plant (B205) to reprocess small quantities of oxide fuel from the early British AGRs and the steam generating heavy water reactor (SGHWR). Up to 1973, 100 tonnes of spent oxide fuel had been reprocessed there. In 1973, however, an accident in B204 resulted in it being shut down. Some radioactivity was released into the operating area and several people were contaminated. BNFL is currently modifying B204 and it should be reopened in 1979.

The chemistry of reprocessing oxide fuel is very similar to that of reprocessing Magnox fuel. But the method is slightly different. This is because of two major differences between the two fuels. Reactors using oxide fuel — the modern reactors — operate at higher power levels and achieve higher burn up of the fissile contents of the fuel than the Magnox metal-fuelled reactors. Irradiated oxide fuel is therefore very much more radioactive than the metallic fuel: the gamma radiation emitted is seven to ten times greater. The two types of fuel also differ in the way they are packaged. Uranium-metal fuel is contained in a thin coat of soft magnesium alloy, or Magnox,



'I was flabbergasted . . . It is incomprehensibly one-sided . . . not a dismissal of our case but a bypass of it' Walt Patterson, Friends of the Earth

and oxide fuel in either zirconium alloy or stainless steel cans.

Because of its lower radioactivity, Magnox fuel can be stored directly in pond water. After pond storage, however, the magnesium alloy is stripped off before the fuel is dissolved in nitric acid. The solvent extraction process is also slightly different. Because oxide fuel is highly radioactive, the process is carried out in pulsed columns (rather than mixer settlers) to minimise the contact time between the solvent and the dissolved acid fuel and so reduce degradation of the solvent. Radiation shielding of all processes involving oxide fuel has to be greater because of the increased radioactivity.

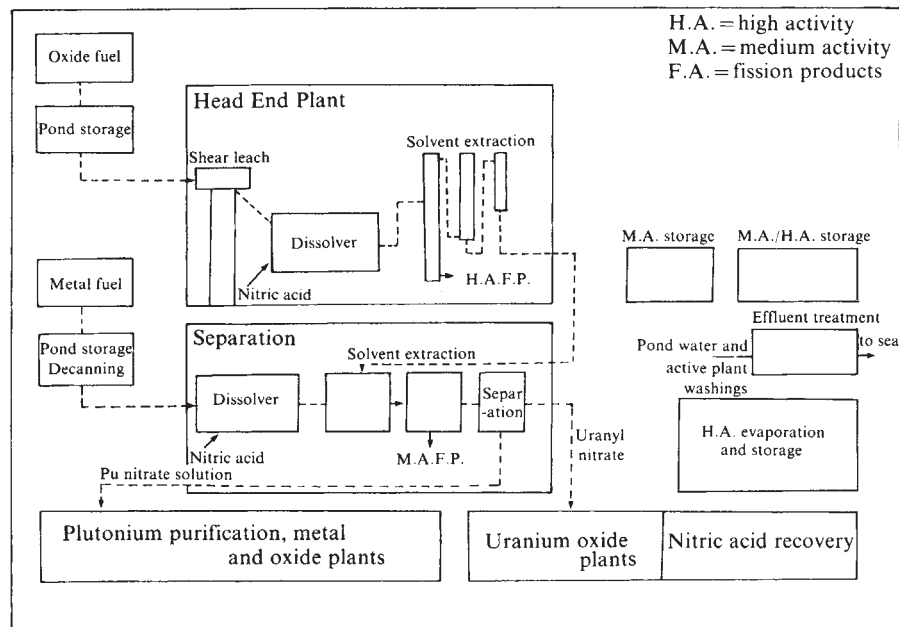
If Parliament votes that THORP should go ahead, BNFL will have to work out a detailed design for the plant. It intends to build a fully operational 1/5000 scale model of the whole plant and full scale models of pulsed columns and mixer settlers for operation with uranium solutions but not highly radioactive material. The design of THORP will be based on these models. No intermediate scaling up will be done.

The design phase will probably last until about 1982; very little of the estimated £600 million cost will have been spent by then. Building should start on spent fuel storage tanks in the early 1980s. These have to be ready well in advance of the reprocessing facility to receive spent fuel, especially that from Japan. BNFL estimates that the cost of building a plant to reprocess foreign as well as British fuel is only 20% greater than the cost of building a plant for British fuel only. The UK generating boards, it claims, would be saved £55,000 per tonne of fuel reprocessed if THORP is built on the basis that half the capital cost is born by foreign customers.

The waste of THORP

The diagram (right) shows the path of spent fuel through a reprocessing plant.

In a typical reactor, roughly 30 tonnes of spent fuel elements would be withdrawn each year, after three years service. On average this spent fuel would contain one tonne of fission products, half a tonne of unburnt uranium-235 and plutonium in equal quantities, and about 0.2 tonne of uranium-236, neptunium, americium and curium. After separation, the fission product solutions have the highest radioactivity and generally also contain some of the very long-lived actinides. The fission-product activity in the 30 cubic metres or so of these solutions decays rapidly, however, and after a few hundred years has virtually disappeared; what remains is the very long-lived but low-level actinide activity.



A black and white report for debate

REACTIONS to Mr Justice Parker's report on the proposed extension to facilities for reprocessing thermal reactor oxide nuclear fuel have varied widely. Arthur Palmer, MP, Chairman of the Select Committee on Science and Technology, for example, called it "an excellent report, far better than anticipated. . . . Parker is extremely realistic—he looks on nuclear power, of which reprocessing is an integral part, as a necessity in an industrially advanced country—as I do". Robin Cook, MP, charges Parker with making selective quotations from his witnesses—and it is true that many objectors are quoted only when they are in favour of some aspect or other supporting the construction of THORP. "I am extremely concerned with the report" said Cook last week. "Parker has decided to present the case for THORP—and he's better at it than BNFL".

Cook, speaking at a briefing meeting for MPs at the House of Commons last week, said there were alternative sources to nuclear power such as coal, oil, gas, waves, tidal barriers, wind and solar energy "and if we cannot squeeze a future out of that it is a very sour comment on our previous energy investment".

Palmer, an electrical power engineer, looks on it very differently. "The report is not afraid to tell the blunt truth" said Palmer. "Even if nuclear power had every risk attributed to it by its critics—which it hasn't—it would still be necessary if this country is to survive commercially and prosper". If there is low nuclear growth, Palmer believes, Britain will lose its nuclear power engineers, and the nuclear industry would be broken.

A debate on the Windscale report takes place in the House on Wednesday 22 March, following a complicated procedural move by the Secretary of State for the Environment, Mr Peter Shore, to avoid the legal complications in such a course. Mr Shore was obliged to take a decision on THORP without hearing further argument: so he refused permission for it to be built, arranged this Wednesday's debate, and is expected to lay a "special development order" before the House to grant permission after Easter. Mr Shore promises to take note of the "feeling". There will probably be no division.

Pressure for the debate came with an 'early day motion'—effectively an MP's petition—some weeks ago requesting a debate; it was signed by 200 MPs.

Whether that number will turn up on Wednesday is not clear at the time of writing. The following day is the last day of the session and is filled with procedural business; and many MPs return to their constituency the day before. The estimate of one MP who hopes to speak in the Windscale debate is that perhaps a dozen MPs in the House are sufficiently well informed to make effective contributions; and that some 40 more are interested enough to speak but have not studied the problem carefully. There should be time in the seven hours of the debate from 3.30 to 10.30 pm for about 12 speakers on each side of the House to speak. A head-count makes it clear that the opposition to THORP, though vocal, is very small; which makes it difficult to interpret Peter Shore's intention to take note of the debate. Will he take note of the points made

and write his special development order accordingly? Or count ayes?

If he looks for spectacular speeches, then he could do no better than take note of Leo Abse, MP for Pontypool, who made a name for himself last December as a new and rhetorical star in the nuclear campaigner's firmament. Nuclear power is Abse's new concern. "One or two debates in the House of Commons do not end matters" said Abse last week. "I am used to long campaigns". Abse has been a long and successful campaigner for many reforms, particularly in the laws relating to private life—such as divorce and homosexuality. It remains to be seen how he fares with the equally emotive but highly technical matters of nuclear energy. Mr Wedgwood Benn, Secretary of State for Energy, congratulated Abse warmly for his December speech on these matters. But another observer, well briefed in nuclear matters, described it as "30% right but 70% rubbish". We shall see. □

Leo Abse, MP, taking up the objectors' cause



Mr Justice Parker and technical fact

Mr Justice Parker, who some time ago handled the Flixborough inquiry into a devastating explosion in a British chemical plant with consummate judicial skill, applied the same talents last year to the proposed construction of THORP (see p298). He was appointed to the Windscale Inquiry, as it became known, perhaps because he had gained experience at Flixborough in inquiries requiring assessment of technical argument.

Therefore, as one might expect, where the arguments concerning the construction of THORP are capable of strict technical analysis Justice Parker's final report — published by the government on 6 March this year — is masterly. At the lowest level of contention, his description of the basic physics and chemistry of the nuclear fuel cycle should be read by anyone wishing to understand the technical basis of nuclear power and the reprocessing of nuclear fuel.

At the next level, Justice Parker's chapters on risk perform a very valuable function in drawing together the data on the effects of routine discharges of radionuclides from Windscale, and on assessing the possibilities of accident. The Inspector even ordered a few experiments. With their agreement he subjected a few regular fish eaters on the coast (where the existing Magnox reprocessing facility discharges low-level liquid waste) to whole body monitoring for the presence of radioactive elements passed down the food chain to the fish and thence to the fish eater. Levels were well below the limits set by the International Commission on Radiological Protection. In such ways Mr Justice Parker allayed some fears about the dangers of Windscale by ascertaining facts.

Parker has also made some positive recommendations for improvement, as in the scientific competence of the Nuclear Installations Inspectorate (NII) to check the designs of THORP were it to be built, and in the constitution of the Windscale local liaison committee. The latter was set up to prepare emergency plans between the local community and Windscale, but, writes the Inspector 'it emerged in evidence that some of those who, in the event of an emergency, would be required to take action under the plan . . . did not even know they had any responsibilities, much less know what these responsibilities were. This was clearly a grave defect . . .'. British Nuclear Fuels Ltd (BNFL) who control Windscale have agreed to act on all Parker's criticisms so local liaison at least should improve shortly.

Politics is involved in the environmental and health risks associated with THORP at least to the extent that being subject to them is not a matter of choice for the local residents; but it seems clear that the risks are not likely to be greater than those

involved with any other industry.

But in almost all the other issues faced by the Inspector complex political judgements are involved. The two central political questions concerned with the construction of THORP are international and national: the effect on the proliferation of nuclear weapons (which we deal with on p302), and the choice of UK energy future. The latter choice offers a number of options quite distinct from the familiar ones of the high growth and low growth future. On one of these Mr Justice Parker heard two days of evidence (outlined below) from Gerald Leach of



Mr Justice Parker: "We will be castigated anyway"

the International Institute for Environment and Development (IIED), but he pays scant attention to this in his report.

The choice of energy future (in Leach's paper it is one of low primary energy reached largely by choice of efficient consumption technology) drastically affects Mr Justice Parker's recommendation to build THORP.

He argues in his conclusions that: first, extra facilities are needed for projected UK arisings of spent fuel after about 1995; second, the best way of dealing with the wastes is to reprocess the fuel; third, that it is better to build the plant early rather than late to gain experience of the technology and avoid possible technical hitches; and fourth, that if we are going to have reprocessing anyway we might as well improve its economics by importing foreign fuel for treatment. (He disposes of the problem of the proliferation of nuclear weapons on the way by a special reading of the non-proliferation treaty.)

Thus the argument depends on the projected UK arisings, which have their origins in a forecast of the Central Electricity Generating Board. This in turn depends on an energy consumption forecast. A forecast used in this way represents a political decision. It selects a particular path among many options.

Parker's words in the report are

illuminating. Considering UK arisings of spent fuel to the year 2000 he first calculates that 4,150 tonnes will have arisen from existing reactors and from those under construction. He goes on "If, as appears likely, reactors to produce a further 4,000 MW per year of electricity are ordered in the near future and begin to operate between 1990 and 1995 they will, by the year 2000, have produced a further 1,700 tonnes of spent fuel. Thus a total of 6,000 tonnes by the year 2000 from UK reactors alone is a realistic forecast". (Another 4,000 MW is a large increase in nuclear power: the total nuclear generating capacity in England and Wales at the end of March last year was 3,462 MW).

It quite genuinely seems not to have occurred to Mr Parker, that the use of "as appears likely" and "realistic" is, intentionally or not, loaded, representing a choice of paths among alternatives. For example, the Department of Energy's recent decision to invest in the conservation of energy is projected by the department to reduce energy consumption by the end of the century by 20%—or the equivalent of about 40,000 MW. Flexibilities of that order by political choice are quite feasible.

Energy futures

Gerald Leach's two days of evidence, which formed an interim report on a study to be published by IIED in June, described a future with "substantial rises in material standards, mobility and other energy-related activities". But it required not a rise but a reduction primary energy demand by the year 2000. This is achieved through increased consumption efficiencies with, for example, gas-fired heat pumps for heating and good thermal insulation. The energy group of the Sussex University Science Policy Research Unit has also discovered that market competition between North Sea gas and electricity at the end of the century might restrict electricity to "essential" uses—such as lights, television, and electric motors. It follows, because of the poor energy efficiency of electricity generation in power stations, that on this basis there is a reduction of some 40% in primary energy consumption.

Futures studies have lately taken on a completely new meaning, of which Mr Justice Parker does not appear to be aware: the meaning of offering an option to a policy maker, not of predicting the future. Mr Wedgwood Benn, the UK Secretary of State for Energy, expressed a view on this at a meeting on Windscale at the Royal Institution last year: "To be mes-

merised by forecasts is a way of getting you to do what the forecaster wants you to do" said Benn. "I want to have elbow room". Elbow room—in the form of a set of decision options and their probable consequences—is what energy forecasts are now offering; it is false thinking to take only one as "realistic".

Mr Justice Parker uses his single set of projected arisings to indicate that the existing Windscale reprocessing facilities, and those already granted planning permission, are insufficient to cope with the load.

Waste management

The next step is to show that the only effective means of dealing with this excess of spent fuel is to reprocess it—that is, to treat it chemically to separate out the highly active wastes and, as a by-product of the existing process, produce separated uranium and plutonium. (The latter is not an essential step in that it can be reversed to produce a degraded mixture of the two, but it is essential if it is wished to conserve plutonium for bombs or fast breeder reactors).

Here again a political element creeps in. The inquiry considered two principal options. The first is as proposed for THORP, to reprocess spent fuel rods, separate out the highly active wastes containing fission products and actinides, glassify the result and dispose of it underground in geologically stable rock formations. The second is to hold the spent fuel in its can, perhaps further encapsulate it, and store it in cooling ponds or in dry vaults (possibly filled with inert gas).

Each means of waste management suffers an unresolved technical problem. In the reprocessing route, we do not know if geological storage will work for the 100,000 years or so for which the actinides are decaying. If it failed, a large amount of radioactivity could be released upon future generations. Equally in the storage route we do not know if the cans (stainless steel in the case of AGR reactors and zirconium alloy in the case of PWRs) will corrode.

Agreed scientific knowledge is lacking in both cases; and projecting the futures of materials must always be contentious as the only conclusive way to test a theory is to wait and see. Effective work on both has only recently been undertaken. What research there has been has concentrated on the reprocessing route, as reprocessing has always been the objective of the nuclear industry for a reason other than waste management: the separation of plutonium.

So it is difficult, if not impossible, to assess in an entirely objective way which is the most effective means of

waste management. In such cases an apparently scientific and technical choice is in reality influenced by other forces: particularly, consideration of the decision one wants to reach. In that sense the choice becomes a matter for political debate. Mr Justice Parker's choice (classification) appeared to rest on his confidence in BNFL, hardly an impartial observer.

Proliferation

The most difficult political judgement in the report concerns the proliferation of nuclear weapons through the separation of plutonium in reprocessing. (In Japan's contract with BNFL, for example, plutonium—and the wastes—ultimately return to Japan, if the US, supplier of the original uranium, is willing.) If energy futures studies and waste management options offer a political choice, the dangers of proliferation require a finely balanced international diplomatic and political perspective, and should surely not have been a matter for decision in a court in Cumbria. BNFL's argument that providing reprocessing facilities, under international control, for foreign countries was depopulating in that it discouraged those countries from developing their own unsafeguarded reprocessing plants was accepted by Mr Justice Parker.

Yet he did not apply to those countries the argument he applied to the UK: that there was pressure to reprocess to lessen dependence on foreign suppliers through the collection of plutonium (which might give a 50-fold increase in the available energy in a given stock of uranium, if fast breeder reactors were employed).

Nor did Parker give weight to the over-riding question in proliferation: the effect that the example of build-THORP might have on the tense international atmosphere prevailing on commercial trading in nuclear materials.

Mr Justice Parker accepted the arguments of BNFL and yet BNFL's strict impartiality on proliferation must be questioned. It is true that many people in the nuclear industry have been involved in international exercises on non-proliferation throughout their careers; but they have inevitably mixed motives.

Their aim is to transfer nuclear technology and materials; but with that goes a small but unavoidable risk of bringing another country closer to nuclear war—a risk they attempt to reduce. One way of reducing the risk (in the short term at least, before the country creates its own technology) is not to transfer the technology or materials at all. But that option would not be likely to occur to them. The

attitude of certain senior members of BNFL to proliferation is, to say the least, equivocal: in an interview with *Nature* last week one made it plain that in his view limited nuclear war in remote countries using weapons made from commercial-grade plutonium need not necessarily escalate to global conflict and would be survivable even for the countries involved.

It is with this kind of pressure—whether those producing it were aware of it or not—that Mr Justice Parker was dealing; yet he shows no signs of recognising the fact in his report, and perhaps compensating for it.

Mr Justice Parker, however, is not to be castigated for coming to decisions on all these issues. That was his role: as a judge, to judge. But while demonstrating the ridiculousness of many of the more irrational fears about nuclear power—a good thing—he did little to illuminate the corners where the political decision making lay.

The Inquiry itself, however, had done just that, and many had been left with a hope—which in retrospect seems no more than romantic—that the report would reflect the debate. But Mr Justice Parker was there to decide, not to illuminate controversy.

Yet it might still be the case that the kind of analysis given here of the political content of technical decision making would be alien to him. Introduced by politically leftist thinkers and groups it seems automatically to have been ignored by those of a rightist inclination—because of its unsavoury associations. But the decisions would still have a political content even if Mr Justice Parker had decided in completely the opposite direction, and this article would have equal force. It is the nature of the case. Technical decisions as complex as these have a political content and that content must be isolated and recognised for what it is.

It is illuminating to discover that, with a few outstanding exceptions, those to whom the political content of the report has become apparent are those who disagree with its conclusion. Sir John Hill, chairman of the United Kingdom Atomic Energy Authority, is delighted with the report for its "lack of ambivalence. I'm not fond of debate" said Sir John "I prefer analysis". Sir John considered the document to be preferable to its forerunner on nuclear power, the sixth report of the Royal Commission on Environmental Pollution, chaired by Sir Brian Flowers, which he did find to be ambivalent. But this indeed was its strength, in that it declared the places where there must be political decisions and proffered the evidence. Mr Justice Parker, if the constitution of the inquiry had allowed it, would have done better to have done the same.

Robert Walgate

The Non-Proliferation Treaty — a requirement to reprocess? NFCE — an exercise in proliferation

In the Windscale report Mr Justice Parker held that there was a case for THORP being large enough to reprocess foreign fuel as this would 'do something to relieve the pressure on non-nuclear-weapon states to develop their own facilities. It would also demonstrate that this country intends to honour at least the spirit, and as I think the letter, of its obligations under the Non-Proliferation Treaty (NPT)'

NPT came into force in 1970 and now has 103 states party to it. These include Japan, West Germany, Sweden and Switzerland but not Argentina, Brazil, France, India, Israel, Pakistan, People's Republic of China and South Africa.

Article I says amongst other things that each nuclear-weapon state party to the treaty undertakes not to transfer to anyone nuclear explosive devices, nor '... to assist, encourage or induce any non-nuclear weapon state to manufacture or otherwise acquire nuclear weapons ...', and Article II is a similar commitment on behalf of non-nuclear weapon states not to make nuclear weapons nor to receive assistance in their manufacture. Mr Parker held that neither article could be taken to prevent the transfer of plutonium, only explosive devices. He also held that transferring plutonium could not be regarded as assisting proliferation because were it so the already considerable exports of plutonium by the UK and US to non-nuclear-weapon states must be regarded as contravening the NPT — which no one claimed at the time.

He further goes on to argue that since Articles III and IV mention peaceful uses of nuclear energy, both in terms of safeguards to prevent diversion to military purposes and in terms of reassurances that peaceful programmes are not meant to be interfered with, it must be assumed that those who framed the treaty recognised the non-nuclear-weapon states' interests in using fissionable material. What is more Article IV enjoins all parties to the NPT to facilitate 'the fullest possible exchange of equipment, materials and information for peaceful uses'. At the time the treaty was entered into, plutonium production by reprocessing was certainly envisaged. So, argues Parker 'I find it difficult to see how a party ... would be otherwise than in breach of the agreement if it ... refused to reprocess for another party'.

Further, Parker goes on to reject claims that if there was an obligation of this nature it could not be imposed if it involved economic loss. 'The NPT is on its face a straightforward bargain', he writes. Loss would be a natural price to pay for restraint by non-nuclear weapon states.

INFCE, the International Nuclear Fuel Cycle Evaluation programme, was first heard of last April when President Carter, in the course of a major policy statement on nuclear proliferation, mentioned that discussions were being conducted with countries exporting or importing nuclear fuel and technology, with a view to developing an international viewpoint on how to keep the dangers of nuclear proliferation out of the nuclear fuel cycle.

Forty countries, IAEA, Euratom and OECD are now involved in INFCE, which had its first meeting last September. There has been strong support for the concept both from east and west; the only notable absentee is the People's Republic of China.

Eight separate study areas have been established:

- uranium supply
- enrichment
- security of supply
- reprocessing
- fast reactors
- spent fuel management
- waste management
- other fuel cycles.

The general philosophy is to explore ideas both concerning technical fixes and also establishing new frameworks for international cooperation.

Group 4, on reprocessing, is of particular interest at present; in it the UK and Japan are co-chairmen. There are two sub-groups — one on reprocessing headed by Dr Walter Marshall (UKAEA), and one on thermal reactor recycling headed by Dr S. Tamiya (Japan Power Co.).

At the present INFCE is much more of a technical evaluation exercise than an international negotiating forum and maybe as a result participants in it are optimistic that its deliberations will lead to a useful outcome. But, say some critics of the Windscale report, if INFCE is doing well and its discussions proceeding apace, why shouldn't BNFL wait a year or two rather than proceed with THORP now? The answer seems to be that participation in INFCE is seen as a means of assessing long-term options, not as a way of affecting short-term decisions.

Japan is a strong supporter of INFCE, and this is seen by many as a way to shake off the tight US controls on the export of enrichment and reprocessing technology.

One of the most serious problems for participants in INFCE is how they prevent a non-proliferation exercise from making proliferation easier. It is difficult to advise a non-nuclear-weapon state what it should do and what steer clear of without bringing the State that much nearer to having a nuclear weapons capability. Nor is it entirely possible to prevent such a

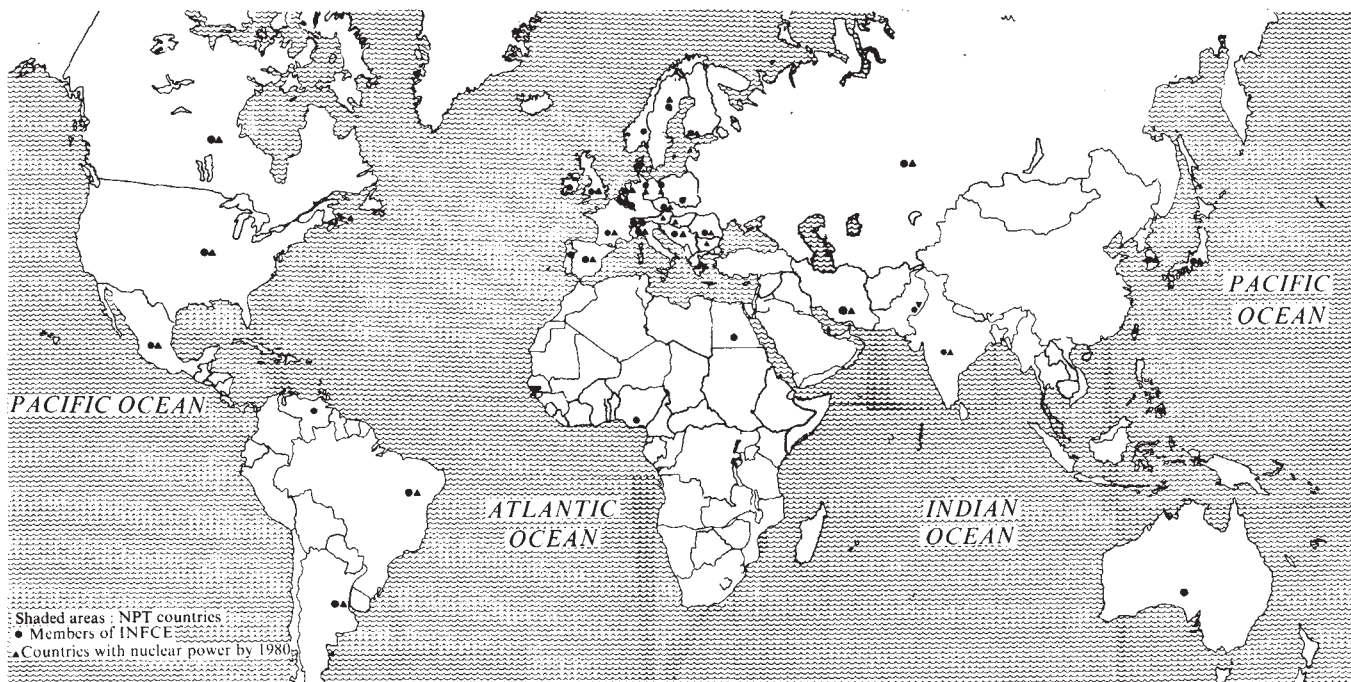
forum from being a place where commercial expertise gets more widely disseminated than many would wish. So the price nuclear-weapon nations may have to pay for participating in INFCE may not be negligible, either politically or commercially.

Swedish experts recommend nuclear energy — without reprocessing

After more than a year's hard labour, the government-appointed Energy Commission has finished its work. Its final report, to be presented to parliament on 27 March, is already completed; and the tenor of its recommendations is clear. The majority of its members are in favour of between 10 and 13 nuclear reactors — the exact number has, according to informed sources, been left unspecified. This means a defeat for the Prime Minister's Centre Party, whose representatives on the Commission have been fighting to stop nuclear energy.

According to the same sources, the report says that reprocessing plants should not be built in Sweden for the time being. It also advises waiting for the results of the International Fuel Cycle Evaluation (INFCE) studies on the handling of spent fuel before making definite choices between different methods of final deposition and storage. It does, however, recommend continuing prospecting for uranium and preparations for some mining; although whether this should be in the fiercely-disputed Ranstad area, maintained by environmentalists to be untouchable, or in the far north, the report evidently does not make clear.

It is not at all sure what will happen after the report has been presented. Prime Minister Fälldin originally set up the Commission as a think tank which would recommend that nuclear power be stopped. This would have given him solid political backing for keeping the most important promise he made to the voters before his government came to power: of backtracking down the road to a nuclear-energy society. But now that the Commission has back-fired, Fälldin has said that he will treat its recommendations as one of a number; and has referred to the fact that the government does not always act according to the guidelines set down by advisory commissions. Whether he will in fact be able to withstand the enormous pressures that will be brought to bear on him by his two coalition partners — both pro-nuclear — is doubtful. He does of course hold the trump card of resignation, bringing the first non-socialist government in 44 years down with him; but such drastic action seems unlikely given his record of compromise — some would



say capitulation — rather than risking the break-up of the coalition.

Why are the Commission's recommendations so different from what Fäldin hoped for? According to one of its Centre Party members, Birgitta Hambræus, the rot set in as soon as the experts were appointed. She says that, although all the members are agreed that Sweden will have to look to renewable energy sources for its long-term needs, the experts were chosen largely from areas concerned with present-day energy production. Expertise on nuclear power outweighed that on, for example, energy forests; and this is reflected in the recommendations. Mrs Hambræus sees this as a problem of democracy: how to introduce the values a society wants to realise into a supposedly value-free examination of all possible courses of action open to that society? Because this was overlooked, she maintains, all the Commission's hard work has led to a report that is not in Sweden's interest at all.

The Centre Party's priorities are reflected in a government bill published this week, which proposed that Sk1 billion (about \$US217 million) be spent on energy research and development over the next three years. The allocations, on a scale about triple the ones for the current budget period, are oriented away from nuclear power (fission would get only about \$US3 million the first year, with undecided sums after that) to renewable energy sources: at least \$US45 million over the three years is proposed for wind, energy forests and peat.

The bill, which will be voted on before 1 July, outlines the three-year budget proposals for six areas:

- more efficient use of energy in industry (forestry, pulp, paper, iron and steel): \$US18.7 million
- transport (collective transport, alter-

native fuels such as methanol, ethanol, synthetic petrol): \$US7 million

- building (insulation, solar heating, experimental buildings): \$US33.7 million
- energy production (reducing dependence on imported oil through developing domestically-available — primarily renewable — energy sources such as wind, peat, energy forests; the technology of using coal; using hot water from industry for heating; safety aspects of fission energy; advanced energy sources such as fusion, solar and tidal power): \$US85.4 million

- studies of future energy production systems: \$US3 million
- basic research for carrying out these programmes: \$US6.5 million.

\$US5 million is proposed for international cooperation (the cost of Sweden's participation in the International Atomic Energy Agency, for example), and \$US20.8 million is kept in reserve for prototypes. A special allocation of \$US34 million is proposed for the state-owned Atomic Energy Company, which the government wants to re-name Studsvik Energy Technology Company to underline the fact that its expertise and resources will in future be used to research and develop all sorts of energy sources rather than primarily nuclear ones.

Presenting the bill, Energy Minister Olof Johansson explained that allocations to the various renewable energy sources had been proposed according to the time-scale on which they were likely to produce results. Thus wind energy, which the government expects could be developed and possibly put fully into operation during the 1980's, would be given \$US23 million; whereas solar energy (as distinct from solar heating), which is not expected to be operating before the turn of the century, would be allocated less than \$US10 million.

Wendy Barnaby

Moves to terminate US fast breeder project may be illegal

PRESIDENT Carter's efforts to terminate the controversial Clinch River fast breeder reactor project at Oak Ridge, Tennessee may be illegal, according to the US comptroller general, Mr Elmer B. Staats.

The president has consistently opposed the project, on the grounds that it could increase the chance of the proliferation of nuclear weapons, and claiming that it is "neither necessary to meet our projected energy requirements nor economically sound."

Last autumn he vetoed an appropriations bill which would have given money to continue the project. And last week, in signing a supplemental bill that included \$80 million for the Clinch River reactor, the president said that the money would be used to terminate the project.

However, Mr Staats, who is head of Congress' General Accounting Office, has written to both President Carter and the Energy Secretary, Dr James Schlesinger saying that this action is illegal.

According to Mr Staats, the legislation signed by Mr Carter required "that the funds available for the project be used only for the design, development, construction and operation of the liquid-metal fast-breeder reactor, and that they may not be used to terminate such activities."

In his letter, Mr Staats said that if the administration decided not to use the money for the purposes set out in the appropriations bill, the federal officials certifying termination of the project "shall be held accountable for and required to make good to the United States the amount of any payment prohibited by law."

David Dickson

The Japanese Connection

£200 million-worth of foreign contracts for reprocessing oxide fuel at THORP have already been signed. Foreign contracts worth £400 million are currently under consideration and are dependent on THORP being given the go-ahead. The largest one in this category is the Japanese deal worth £300 million. Most of the remaining £100 million-worth of foreign contracts waiting for signatures are European.

BNFL plans cautiously to use about half of THORP's total reprocessing capacity of 1,200 tonnes of spent fuel per year. 300 tonnes of the 600 tonnes it will reprocess yearly will be British and 300 foreign.

The Japanese contract is for reprocessing about 160 tonnes of spent fuel per year for between ten and thirteen years.

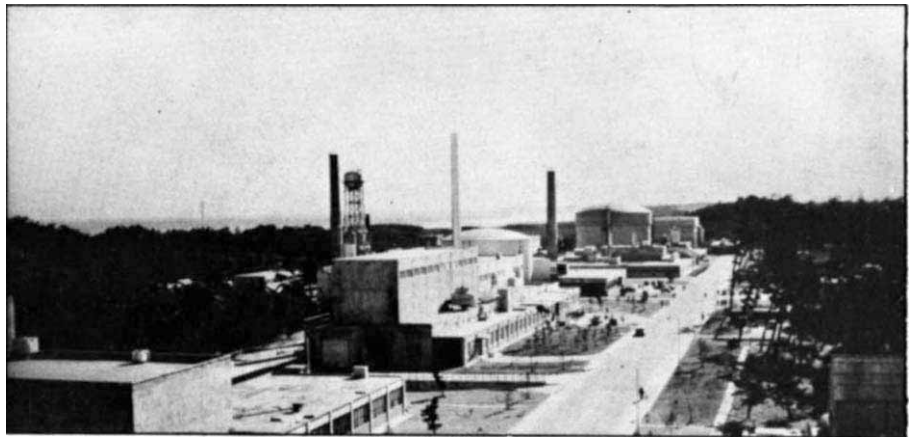
At the Windscale Inquiry, Raymond Kidwell, Counsel for the objectors, and Brian Silsby, Counsel for the applicants, agreed on a summary of the Japanese contract which could be made public. The main points of their summary are as follows:

- Customers will contribute towards the building of THORP by advancing a fee based on the proportion of the available reprocessing capacity they wish to reserve. BNFL will be paid at cost, whatever that may be, plus a margin of profit. Cost will cover construction and operation of the plant and the return of wastes or unprocessed fuel.

- The technological and operational risks do not fall on BNFL.

- BNFL has the option to return any waste which is in a form suitable for safe transportation and storage. And reprocessing will not begin until BNFL is satisfied that a process exists which could make waste safe for transportation and storage. The Japanese, however, will only accept glassified waste so if that process were not glassification BNFL could stop reprocessing. If glassification were later developed, wastes could be returned to Japan within 25 years of reprocessing ceasing. If glassification is never developed or fails, BNFL can ultimately return unprocessed fuel. BNFL would then be obliged to receive contractual quantities of fuel up to 1990 and return it by 1995. The same applies if reprocessing never begins or is permanently halted.

- BNFL would only have to repay any money received if reprocessing did not take place and BNFL earned revenue by making facilities available to another customer. The original customer would then be refunded out of the earnings.



- No contract would be signed without the government's consent. If an international agreement is not reached within 90 days of the contract being signed, BNFL can void the contract.

- The transfer of nuclear material under contract is subject to international safeguards and regulations referring to the retransfer to countries other than Japan and that it shall only be used for peaceful purposes. The British government 'requires to be consulted' before plutonium, or uranium enriched in uranium 235 above 20%, is re-exported to a third country.

- When BNFL announces that plutonium is available to a customer it must be stored for a minimum of five years or until the customer needs it for fuel fabrication. After five years, the customer can receive plutonium without stipulating that it is to be used for fuel fabrication.

Japan is burning to be nuclear

Japan has very little in the way of indigenous energy: except for the odd earthquake and the tsunami — not the softest of renewable energy resources. So it is highly dependent on energy imports — among them uranium bought mostly from the United States. To free herself somewhat of this dependence Japan is committed herself to a plutonium future of fast breeder reactors and — inevitably for fast breeders — reprocessing to produce the plutonium. Japan already has a small reprocessing plant at Tokai Mura, recently come on line by agreement with the US.

The US is putting increasing pressure on Japan to conform with its strict guidelines of nuclear propriety — and equally Japan is trying to slip round them. Thus Japan strongly supports INFCE (see previous page) as a means of internationalising controls.

According to the *Japan Times* last week, the chairman of the Japan Atomic Industrial Forum, Hiromi Arisawa said recently "all nations should await the

studies of INFCE". The paper commented that this remark implied criticism of American moves to place tight controls on the export of nuclear power unilaterally.

Three years ago Japan planned by 1980 to have 320 tonnes per year of spent LWR fuel to reprocess, and to put 60% of its reprocessing abroad. (Windscale has a fair proportion of that under an existing contract lasting to 1985). By 1985 Japan planned 470 tonnes, 69% abroad, and by 1990 a massive shift to 990 tonnes, only 35% of which was to be reprocessed abroad.

Japan is also attempting to design a 1500 tonne per year reprocessing plant to come on line in 1990 — not to take its business away from British Nuclear Fuels Ltd or its other contracted reprocessor, COGEMA of France — but to help reprocess the large amount of nuclear fuel it projects to have lying around by that time.

This clearly took into account a large reprocessing facility within Japan; but the US are proving sticky negotiators.

Presently Japan has 14 reactors producing power — 13 light water reactors and one gas cooled reactor — providing between them some 8000 MW. The present target for 1985 is 26,000 MW; but the government has said that "with maximum effort" Japan could install 33,000 MW. The Liberal Democratic Party — the ruling party — says that target should be met, though informed observers think that 22,000 MW is more likely. MITI, the Ministry for International Trade and Industry, are even more optimistic for 1990 with a projected "maximum effort" capacity of 60,000 MW. Beyond that the Japanese are pressing for a full fast breeder reactor system.

The Tokai Mura design capacity is 200 tonnes per year though it believed so far to have only processed kilogram quantities. With the US, the Japanese are studying the feasibility of coprocessing at the plant — a method of producing a mixed output stream of uranium with plutonium, instead of the existing "PUREX" output of separated plutonium and uranium nitrates.

news and views

Hypercomplexities in the visual cortex

from J. Anthony Movshon

In their pioneering studies of information processing in the visual cortex of cats and monkeys, Hubel and Wiesel (*J. Physiol.* **160**, 106; 1962; *J. Neurophysiol.* **28**, 229; 1965; *J. Physiol.* **195**, 215; 1968) distinguished three major classes of neurone by recording single-unit responses to slits, edges and spots of light. 'Simple' and 'complex' cells are selectively sensitive to the orientation and, often, the direction of movement of visual contours; they differ from one another in details of the organisation and size of their receptive fields. 'Hypercomplex' cells add to orientation and direction selectivity a preference for the length of stimulating visual contours: their responses are reduced or abolished by elongating an optimally-oriented contour beyond a limited area of the receptive field. Originally, the properties of hypercomplex cells were thought to be generally similar to those of complex cells. Their excitatory inputs were held to derive from one or a few complex cells with superimposed receptive fields, while their length-specificity was thought to result from the inhibitory influence of other complex cells with displaced receptive fields.

The need to modify this scheme was first suggested by Dreher (*Invest. Ophthalmol.* **11**, 355; 1972), who found that hypercomplex cells in the primary visual cortex (area 17) could be divided into two groups according to their general similarity to either simple or complex cells. Both groups share the defining property of hypercomplex cells—length specificity—but otherwise resemble one or the other of the principal cell types. The clarity of the situation was further reduced by the observation in a number of laboratories studying both cat and monkey visual cortex that it is difficult on quantitative grounds to justify a separation of one group of cortical cells on the basis of length specificity alone. The extent of the reduction in response with increases in stimulus length varies for

all cell types, and the distribution of length-specificity is not obviously bimodal (Schiller *et al. J. Neurophysiol.* **39**, 1288; 1976; Gilbert *J. Physiol.* **268**, 391; 1977; Rose *J. Physiol.* **271**, 1; 1977).

Nonetheless, it is possible to distinguish a group of cells (which may or may not form a truly separate class) whose responses are totally or nearly totally abolished by increases in stimulus length. These neurones, which are most often found in the superficial layers of the visual cortex (Gilbert *J. Physiol.* **268**, 391; 1977), have recently been studied in the cat by Sillito and his colleagues, using a combination of electrophysiological and pharmacological techniques (Sillito & Versiani *J. Physiol.* **273**, 775; 1977; Sillito *J. Physiol.* **273**, 791; 1977).

Sillito uses a multi-barrelled micropipette electrode; electrical activity can be recorded from single neurones through the central barrel, while the additional barrels (usually four) are used for the iontophoretically-controlled microapplication of specific drugs to the region near the electrode tip (this area is roughly 100 μm across, and thus usually includes the neurone being recorded from but few, if any, others) (Hess *et al. Expl Brain Res.* **22**, 415; 1975). The most interesting of the agents used by Sillito is bicuculline, which antagonises the action of γ -aminobutyric acid (GABA), a substance known to act as an inhibitory neurotransmitter in the visual cortex (Iversen *et al. J. Physiol.* **212**, 519; 1971; Sillito *J. Physiol.* **250**, 287; 1975). By comparing neuronal responses to visual stimuli in the presence and absence of bicuculline, it is possible to infer the contribution of excitatory and inhibitory mechanisms to particular receptive field properties. While these techniques are to some degree imperfect (two problems being the uncertain diffusion of the drugs and the possible action of bicuculline-resistant inhibitory neurotransmitters), Sillito has previously demonstrated their usefulness in studies of the factors influencing orientation and direction selectivity in the visual cortex (*J. Physiol.* **250**, 305;

1975; *J. Physiol.* **271**, 699; 1977); the results of his studies of hypercomplex cells are in many ways more striking.

Under Hubel and Wiesel's original scheme, abolishing the inhibitory inputs to a hypercomplex cell should leave its orientation and direction selectivity unaltered, since these properties should derive from the excitatory influence of a few complex cells. The cell's length-specificity, which should be derived from inhibitory influences, would be expected to disappear. Sillito's results are in almost direct contradiction to this prediction: he finds that the application of bicuculline totally abolishes hypercomplex cells' orientation specificity, while only partially altering their length specificity; direction specificity is apparently unaffected by bicuculline. In other experiments, involving the application of D,L-homocysteic acid (which artificially induces a spontaneous discharge in normally silent cells, against which reductions in firing rate can be observed), Sillito demonstrates that at least a part of hypercomplex cells' length specificity is due to active inhibition at some stage in the visual pathway, but his overall results suggest a very different scheme from that of Hubel and Wiesel.

It seems that hypercomplex cells must receive their excitatory input from cells lacking orientation selectivity, but possessing some degree of both direction and length specificity. Sillito suggests that the neurones responsible may be the large pyramidal neurones located deeper in the cortex, which are known to project primarily to subcortical visual structures but which often send recurrent collateral projections into the upper cortical layers. These neurones are a special subgroup of complex cells lacking pronounced orientation selectivity but having both direction selectivity and a preference for shorter stimuli (Palmer & Rosenquist *Brain Res.* **67**, 27; 1974; Gilbert *J. Physiol.* **268**, 391; 1977). Under this scheme, two of the basic properties of hypercomplex cells—direction and length specificity—would in large part be due to the properties of their excitatory inputs, while their orien-

tation selectivity would be provided and their length specificity enhanced by intracortical inhibitory inputs. This model is not entirely satisfactory in that hypercomplex cells generally have rather small receptive fields while the deep-layer complex cells proposed as their prime excitatory input have very large receptive fields, but it nevertheless forces a reevaluation of current thinking about interneuronal interactions in the visual cortex. Another unresolved problem posed by these results is the degree to which the inhibitory influences identified in hypercomplex cells may be identified with the rather generalised and potent intracortical inhibitory influences identified using various techniques (Blakemore &

Tobin *Expl Brain Res.* **15**, 439; 1972; Bishop *et al. J. Physiol.* **231**, 31; 1973; Creutzfeldt *et al. Expl Brain Res.* **21**, 251; 1974; Hess *et al. Expl Brain Res.* **22**, 415; 1975).

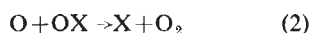
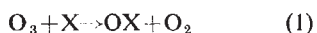
In any case, it is becoming increasingly difficult to conceive of the visual cortex as a straightforward relay system of neurones interconnected by hierarchically ordered excitatory connections which determine the bulk of neuronal properties there. Rather, it is clear that the visual response properties of most neurones depend on subtle interactions within an intricate system of more and less specific excitatory and inhibitory influences, whose cooperative details determine most aspects of neuronal behaviour. □

Ozone increase from Concorde operations?

from Peter Fabian

IN recent years there has been much concern about a possible depletion of the atmospheric ozone layer by man-made pollution. The injection of nitric oxide into the stratosphere by a fleet of supersonic transports or by nuclear weapon explosions, the liberation of chlorine from aerosol propellants and halogenated solvents released into the atmosphere, and the increase of biospheric nitrous oxide emission due to man's increased use of nitrogen fertilizers have been recognised as potential threats to the Earth's ozone shield likely to reduce ozone and thus increase the ultraviolet radiation penetrating to the biosphere.

Ozone is depleted through catalytic cycles converting 'odd oxygen' O_3 and O to inactive oxygen molecules. Their basic chain mechanisms can be written simplistically as



yielding nett



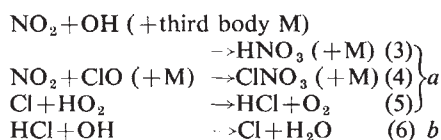
(see inner part of the figure).

X here stands for a catalyst which in the stratosphere can be NO, H, OH or Cl. Any natural or man-made increase of these catalysts may thus deplete ozone. However, this change in ozone concentration depends not merely on the rates for reactions (1) and (2) for

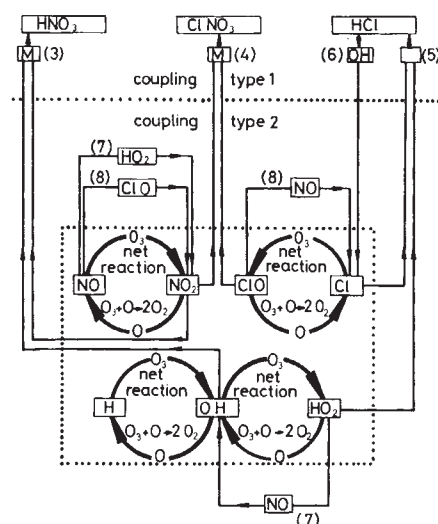
the respective species X. It also depends on the rates of other processes forming and removing X or XO and on reactions interconverting X and XO without ozone depletion.

In fact, the catalytic cycles are strongly coupled, with basically two types of coupling occurring (see figure).

In the first type, the reaction of catalysts from different cycles forms (a) or removes (b) inactive species, such as HNO_3 , $ClONO_2$, and HCl . For example



In the second type, the interconversion of X and XO occurs through reactions with a catalyst from another cycle with-



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out ozone depletion. For example



Any assessment of man's impact on the ozone layer has to take into account the whole chemical system. Due to the coupling processes mentioned the increase of a certain catalyst need not necessarily result in an increased ozone depletion through this particular cycle. In certain instances the equilibrium of other catalytic cycles might be shifted such that even an increase in ozone might occur.

Recently, B.A. Thrush (*Aspects of the chemistry of ozone depletion, Phil. Trans. Roy. Soc.* (in press) given at a meeting of the Royal Society on 'Pathways of Pollutants in the Atmosphere') reported a new rate constant measured for the reaction



determined by J. P. Burrows, G. W. Harris, B. A. Thrush and J. P. T. Wilkinson. In this work nitric oxide was added to HO_2 radicals produced by a Tesla discharge through hydrogen peroxide or by reaction of fluorine atoms with hydrogen peroxide. Using laser magnetic resonance spectroscopy for the detection of HO_2 they obtained the rate coefficient

$$k_7 = (8.2 \pm 1.2) \times 10^{-12} \text{ cm}^3 \text{ s}^{-1}$$

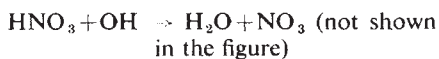
which is about 20 times the value presently used in stratospheric modelling experiments. According to Thrush this much higher rate constant was also confirmed by independent measurements at other laboratories. It has a profound effect on pollution chemistry and ozone depletion, since more HO_2 is converted to OH , and more NO is converted to NO_2 without depleting O and O_3 , respectively (see figure).

For the chlorofluoromethanes, for which the maximum ozone depletion is predicted to occur above 30 km altitude, this higher rate constant is likely to result in substantially higher ozone depletion rates than previously estimated: higher OH concentrations reactivate more Cl from the inactive HCl reservoir (reaction (6)); on the other hand the reduced NO concentrations weaken reaction (8) which converts ClO to Cl without depletion of atomic oxygen (see figure). Thus the catalytic Cl - ClO -cycle gets more efficient, yielding ozone depletion rates about 50% higher than previously calculated according to Thrush.

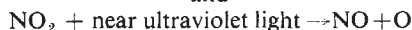
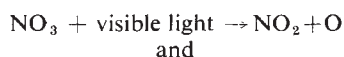
The new rate constant is likely to reduce predictions of catalytic ozone depletion by oxides of nitrogen. Increased NO_2 and OH concentrations immediately lead to enhanced formation of inactive HNO_3 through reaction (3), thus reducing the active species in the NO - NO_2 -cycle. For NO release above 20 km this effect seems small, because HNO_3 formed at

these altitudes by reaction (3) is mainly removed by photolysis.

Thus recent estimates of ozone depletion from NO_x injected above 20 km are not likely to be much changed. For nitrogen oxides from Concorde and high altitude subsonic aircraft, such as the Boeing-747 SP, operating at altitudes up to 18 km, however, Thrush said that a slight ozone increase rather than a depletion might be expected. At these altitudes photolysis of nitric acid becomes less effective. Thus higher OH concentrations make the reaction



compete significantly with photolysis of HNO_3 . Subsequent photodissociation processes such as



lead to the production of atomic oxygen and hence increased ozone. Increased NO_2 concentrations are likely to yield additional oxygen atoms through photodissociation.

Thrush pointed out that more laboratory studies, particularly of other poorly-understood reactions involving HO_2 might well yield more surprises. □

Light in the middle of the tunnel

from Michael T. Flanagan

THE study of the molecular mechanism of active transport across biological membranes has followed the general pattern evolved in the study of the mechanism of action of the soluble enzymes. In this it is hoped that structural studies and kinetic studies will, when they become detailed enough, fruitfully fuse leading to a clear and precise model. Unfortunately the signals monitored in kinetic work, for example, fluorescence emission, often have such low structural information content that they cannot be unequivocally identified with a local rearrangement and the signals monitored in high resolution structural work, such as X-ray diffraction, take too long to collect to be of use in characterising transient species. In the case of intrinsic membrane proteins there is the added difficulty that it is

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Abnormal conditions, normal earthquakes

from Peter J. Smith

THE point (or more accurately the zone) at which three tectonic plate boundaries meet is known as a triple junction. At about 25°S , 70°E in the central Indian Ocean, for example, the African, Indian and Antarctic plates come together to form a triple junction of the ridge-ridge-ridge type at which the three boundaries are all oceanic ridges with, in this case, three quite different spreading rates. Triple junctions are evidently complicated features at which the normal ridge thermal structure and stress fields may be modified. They may also be unstable, changing their positions in response to the three-plate interaction. But what effect, if any, does this complexity and/or instability have on the earthquakes generated in the vicinity of a triple junction? Are the source characteristics of near-junction earthquakes comparable to, or demonstrably different from, those of shocks elsewhere on the ridge system?

To find out, Solomon *et al.* (*Geophys. Res. Lett.* 4, 597; 1977) have monitored microseismicity near the Indian Ocean triple junction with an ocean bottom seismometer. During the two weeks of observation 38 earthquakes were detected, fairly evenly distributed in time; and of these, 17 were local events within epicentral distances of 5–40 km and with local magnitudes (M_L) in the range 0–1.5. For each of the 17 earthquakes, seismic moment and equivalent fault length were deduced from the S wave amplitude spectrum.

To discover whether or not the triple junction microearthquakes were anomalous, Solomon and his colleagues then plotted seismic moment against inferred fault length (log-log plot) not just for these events but also

for microearthquakes on the Rivera fracture zone (Prothero *et al.* *Nature*, 262, 121; 1976), the March 1969 earthquake swarm in the Gulf of California (Thatcher *J. geophys. Res.* 77, 1549; 1972), large ridge crest earthquakes (Solomon *Eos*, 57, 954; 1976), large transform fault earthquakes (Burr & Solomon *J. geophys. Res.* in the press) and one or two other events. In spite of the wide range of data (4 orders of magnitude in fault length, 11 in seismic moment) the data points lie remarkably close to a straight line. There is some scatter about the imaginary regression line, and there is a suggestion of deviation from linearity at the large-earthquake end of the spectrum. But there are no large deviations from the general trend, and the triple junction earthquakes are no exception.

In other words, "the triple junction earthquakes have moments and fault lengths which scale as if the events occurred on a normal spreading center system"; and so, surprisingly perhaps, "the character of the earthquakes near the RRR triple junction in the central Indian Ocean is not demonstrably different from that on other spreading center boundaries". The inference that Solomon *et al.* draw is that any anomalies in the thermal structure or stress fields associated with the triple junction must be confined either to depths greater than that within which the earthquakes are generated or to a small zone surrounding the junction of no more than a few tens of kilometres across.

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often impossible to obtain the required degree of molecular order necessary for high resolution studies. One significant exception is the light-dependent proton pump, bacteriorhodopsin, which naturally forms two-dimensional crystals, a property elegantly exploited by Henderson and Unwin in their revolutionary work on the use of unstained samples and image processing in electron microscopy. This protein is equally well behaved spectroscopically and has over the past few years been the subject of a technique that promises

to bridge the structure-kinetics gap, resonance Raman spectroscopy.

Bacteriorhodopsin is a small protein spanning the membrane. Its chromophore, retinal, is attached to a lysyl side chain as a Schiff base. When light is absorbed by the retinal a photochemical cycle is initiated in which several transient species, with different absorption properties, have been identified and characterised kinetically. During the cycle a proton is released from the external surface of the membrane and one taken up at the cyto-

plasmic surface. The retinal, if extracted from the light-adapted protein, is found to be the all-*trans* isomer and it has been assumed that this is the conformation in the protein. However, in the dark, the retinal slowly relaxes to the 13-*cis* isomer suggesting that it may be in a strained configuration. This possibility is consistent with the position of the absorption maximum of the light-adapted protein (R_{570}), far to the red of simple model compounds, indicating strong local interactions between the chromophore and the protein. Early resonance Raman studies (Mendelsohn *et al.*, *Can. J. Biochem.* **52**, 774; 1974) confirmed that the π -electron density of the retinal is perturbed. Lewis *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **71**, 4462; 1974) were also able to show, using resonance Raman spectroscopy, that the Schiff base in R_{570} is protonated but that in the intermediate, M_{412} , formed after about 40 ms, it is unprotonated. Recently several time-resolved Raman spectroscopic studies have appeared covering the nanosecond (Campion *et al.* *Nature* **265**, 659; 1977), microsecond (Marcus & Lewis *Science* **195**, 1328; 1977) and millisecond time ranges (Terner *et al.* *Proc. natn. Acad. Sci. U.S.A.* **74**, 5212; 1977). Marcus and Lewis established the deprotonation kinetics and Terner *et al.* demonstrated, in a comparison of bacteriorhodopsin spectra with those of model compounds, that the retinal in neither R_{570} nor M_{412} is in the all-*trans* conformation. They claim that R_{570} possesses some features more characteristic of 13-*cis* retinal. This conclusion, as Terner *et al.* acknowledge, relies on a comparison that is weakened by the very different absorption properties of the protein and model compounds. More complex model compounds that absorb in the green, are now being prepared and a further, if somewhat long term, attraction of resonance Raman spectroscopy is that it may be possible eventually to refine theoretical calculations to a degree that removes the total dependence on such comparisons. Warshel (*Nature* **260**, 679; 1976) has already suggested ways in which this might be done in the case of rhodopsin. His caution over their general applicability in probing ground state phenomena, as resonance Raman intensities depend on the change in geometry on going to the excited state, is justified. However, in the case of a process which naturally involves excited states, such as the bacteriorhodopsin cycle, this aspect of the technique may be an advantage. But, whatever difficulties in interpretation may accompany these results, they are clearly more exciting than a confirma-

tion of a relaxed *trans* configuration in that they at least point to a way in which the nature of the coupling between the retinal photochemistry and the proton flow through the protein may be elucidated.

The appearance of similar information about the groups involved in the protein may have to await advances in the technology of ultraviolet-tunable lasers. When it does arrive it will have to be correlated with information now being obtained from direct potentiometric measurements on bacteriorhodopsin. Skulachev's group (*FEBS 11th Meeting, Copenhagen*, **45**, Symp. A4, 49; 1977) has increased the resolution of such measurements to 0.3 ms. They associate with the formation of M_{412} the build up of a third of the transmembrane potential and with the reformation of R_{570} from M_{412} the completion of this potential. They also observe an initial small reverse poten-

tial which they speculate may be associated with the formation of the very early intermediate, K_{590} . Their bacteriorhodopsin is attached to a phospholipid impregnated collodion membrane in the form of proteoliposomes. This technique, though both powerful and convenient, does not allow an easy determination of the arrangement and stoichiometry of the lipid and protein. Hwang *et al.* (*J. Memb. Biol.* **36**, 115; 1977) have overcome this problem by adopting the old technique of Blodgett in which they prepare, on a solid support, multilayers from a well defined monolayer of lipid and bacteriorhodopsin formed at a water-air interface. They are now in a position to make potentiometric, spectroscopic and kinetic measurements on the same sample and have already shown that the decay of M_{412} is inhibited as the layer is dehydrated linking this step with the uptake of a proton. □

Snips in secreted proteins

from M. J. Geisow

MANY proteins exported by cells are initially synthesised as biologically inactive precursors. The active form is ultimately produced from these as a result of processing by proteolytic enzymes. This sequence of events represents a unique form of biological control, since each activation step is highly specific and essentially irreversible. Enzymic activation of precursors can occur extracellularly (as with pancreatic zymogens), at the cell surface (viral protein precursors) and within the cell (conversion of prohormones to hormones, for example). Recent work is beginning to clarify the molecular events involved during the intracellular processing of secreted proteins and to highlight interesting similarities between diverse secretory systems.

Signal sequences

Many secreted proteins whose mRNA molecules have been isolated are found to be synthesised with an amino-terminal prefix of 15–30 amino acids, when their messengers are translated in cell-free systems. These so-called 'signal sequences' (Blobel & Dobberstein *J. Cell Biol.* **67**, 852; 1975) always contain a string of non-polar amino acids and may initiate attachment of protein, ribosome and messenger to the membrane of the rough endoplasmic reticulum (RER).

The growing polypeptide chain is then translocated from the cytoplasmic site through the RER membrane *en route* to its eventual packaging into a secretory vesicle in the Golgi apparatus. Before the nascent protein is released from the RER a peptidase removes the 'signal peptide' but, although this is obviously a key event, the nature, location or even the precise specificity of the enzyme or enzymes are unknown.

Habener and coworkers have shown, using parathyroid gland, that the signal peptidase is probably membrane bound and unable to cleave the 'pre' portion of parathyroid hormone when its precursor is simply added to a membrane preparation (Habener *et al.* *Biochemistry* **16**, 3910; 1977). In the case of prelysozyme, Palmiter *et al.* (*J. biol. Chem.* **252**, 6386; 1977) have shown that the prepeptide is removed before synthesis is very advanced. However, these authors have also demonstrated (Palmiter *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 94; 1978) that another egg protein, ovalbumin, is not synthesised as a preprotein, but simply with the amino-terminal initiator, methionine. Since the same class of cells is involved, it seems probable that alternatives to the currently accepted export hypothesis must exist.

Further processing

For numerous secreted proteins, the removal of the 'pre' extension seems to

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be merely the first step in a chain of proteolytic events. From extracts of secretory tissue, higher molecular weight prohormones can be isolated in addition to the more familiar forms. The additional peptide material is not confined to the amino-terminus, but can also occupy carboxyl-terminal or central positions. Insulin and, possibly, relaxin arise from the excision of a central propeptide. Precursors generally have only a fraction of the biological activity of the hormones themselves. However, it is very unlikely that the additional amino acids are present merely to protect the cells from their own hormonal products, since the appropriate receptors are on the outer plasma membrane surface.

It was pointed out some time ago that the removal of the 'pro' extensions of hormones must coincide with the migration and fusion of storage granules with the plasma membrane (exocytosis), since prohormones are not released. An attractive mechanism linking prohormone processing with exocytosis has been reported for hepatocyte vesicles containing proalbumin (Judah & Quinn *Nature*, **271**, 384; 1978). These authors have shown that the conversion of proalbumin to albumin within Golgi vesicles *in vitro* is conditional upon vesicle fusion. The enzyme responsible is similar to the lysosomal protease cathepsin B. In related studies it has been demonstrated that the microtubule-disrupting alkaloid colchicine, which blocks the secretion of many hormones also prevents the conversion of proalbumin and parathyroid hormone. A plausible explanation of these observations would be that converting enzyme and prohormone are present in different vesicles. Upon fusion, conversion occurs and the joint secretory vesicle acquires some characteristic which marks it out for exocytosis.

Judah and Quinn noted that the conversion of proalbumin, and hence the vesicle fusion event, was dependent on the presence of calcium ions. This story has a familiar ring, but the new idea is that conversion, in the hepatocyte at least, might be yet another process under humoral control mediated through the classical receptor-secondary messenger system, which produces a rise in calcium concentration within the cell.

Processing sites

The nature and location of cellular converting enzymes has been an area of extensive investigation since the discovery of proinsulin. D. Steiner at the University of Chicago and others have suggested that secretory protein precursors are processed at a recognition sequence comprising two basic amino acids (arginine or lysine).

The latter are subsequently excised from the proteins by a second enzyme acting in concert. Proalbumin, parathyroid hormone, proinsulin, proglucagon and progastrin are cleaved exclusively at such sequences. This hypothesis is very attractive in terms of the cellular economy it represents. However, one must balance these observations against the recently reported chicken proalbumin sequence (Rosen & Geller *Biochem. biophys. Res. Commun.* **78**, 1060; 1977). The presence of a single arginine immediately before the albumin sequence indicates that a double basic sequence is not obligatory for processing in this case. Furthermore, several of the hormones mentioned retain unprocessed double basic sequences after release from their precursors.

These apparent reverses for a universal converting enzyme hypothesis may turn out not to be too serious. Mild treatment with pancreatic trypsin alone will release biologically active peptides from most precursors, although not always in the forms normally isolated. This is true not only for prohormones but also for vitogellin—the precursor of the egg-yolk proteins, phosvitin and lipovitellin (Christman *et al. Biochemistry* **16**, 3250; 1977). Trypsin will potentially cleave any basic peptide, so the anomalously high specificity of its action on protein precursors implies that sites at which processing occurs are uniquely exposed to proteases. This conformational requirement for processing could reconcile the current observations on prohormone processing, without the need to introduce individually-tailored converting enzymes.

Multihormones

The potential biological function of large peptide fragments released from prohormones has long been an issue of interest and conjecture. In some instances these balancing peptides are larger than the hormones themselves. The notion that multiple biological activities might be present in the same polypeptide chain has been some time in gaining acceptance in the hormone field. However, precedents exist in the form of multiple functional domains in proteins like IgG as well as in the post-translational cleavages of viral proteins. At present great interest centres on the relationships between the various peptides with opiate-like activity (the endorphins) and some pituitary hormones. For example, D. Smyth and his colleagues have shown that the 'throwaway' portions of pituitary prohormones are not degraded and are present in substantial amounts in pituitary tissue. Looking at these throwaway pieces they discovered an opiate-like C-fragment

(also called β -endorphin by other workers) of the pituitary hormone β -lipotropin, and showed that it was produced by processing at a paired basic amino acid sequence (Bradbury *et al. in Polypeptide Hormones: Molecular and Cellular Aspects, Ciba Foundn Symp.* **41**, 61; 1976). Mains and coworkers have shown that the 31 K ACTH precursor in pituitary cells contains both ACTH and β -endorphin (Mains *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 3014; 1977), and more recently, Mains and Eipper (*J. biol. Chem.* **253**, 651; 1978) have shown that this precursor is cleaved in pituitary tumour cells to produce a shorter ACTH precursor and a β -lipotropin-like molecule from which a β -endorphin-like molecule can subsequently be cleaved. □

Genetic variants in 2-D

from Yvonne Edwards and Dallas Swallow

ELECTROPHORESIS is a method without equal for the detection of individual variation arising from base pair substitutions. It has the potential for detecting about a third of all single base mutations—namely those which give rise to amino acid substitutions resulting in a change of net protein charge. The art of genetic screening by electrophoresis has evolved rapidly since the recognition in the 1940s of the variant form of haemoglobin, HbS, by paper electrophoresis. Major advances were marked by the introduction of media with improved resolving power such as starch and acrylamide, and by the success of enzyme staining *in situ* after electrophoresis, which in its present state of sophistication allows the analysis of virtually all classes of enzymes (Harris & Hopkinson *Handbook of Enzyme Electrophoresis in Human Genetics*, North-Holland, Amsterdam, 1976).

The recent introduction of high resolution protein mapping for the analysis of complex mixtures of proteins (O'Farrell *J. biol. Chem.* **250**, 4007; 1975) may herald a further step forward in the search for genetic variation. This two-dimensional separation technique depends on two independent parameters, isoelectric point in the first dimension and molecular size in the second. This results in excellent resolution of component proteins and is potentially able to detect and dis-

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tinguish any mutational event which produces polypeptides differing from the usual molecule in charge or size. Further, the use of a nonspecific protein stain allows the detection of products of many gene loci in a single procedure.

In a recent paper Anderson and Anderson (*Proc. natn. Acad. Sci. U.S.A.*, **74**, 5421; 1977) have explored the potential of the 2-D method in the analysis of a well characterised group of proteins, the human plasma proteins. Despite the problems associated with the overwhelming presence of albumin these authors have succeeded in resolving around 300 protein zones. Many of these seem to be secondary forms generated by post-translational attachment of carbohydrate side chains, in particular sialic acid, so that the entire pattern is derived from only 75 to 100 gene products. Since several of the serum proteins are obligatory heteropolymers, (for example, immunoglobulin) the number of proteins from which these polypeptides are derived is probably even less. However, the resolution of the constituent polypeptides is superior to that achieved by other methods. Thirty to sixty bands can be separated in non-dissociating electrophoresis (Wright *et al. Clin. chim. Acta* **32**, 286; 1971). Furthermore, because both dimensions are carried out in dissociating conditions it is possible to tell in heteropolymeric molecules which of the constituent polypeptides is affected by the mutation.

Using purified protein and immunoprecipitation techniques Anderson and Anderson have identified more than 30 of the component polypeptides and have recognised several well known polymorphisms as well as rare allelic forms of the arginine-rich lipoprotein and an unidentified protein. The complication of the electrophoretic pattern by heterogeneity due to sialic acid could be minimised by treatment of samples with neuraminidase before electrophoresis, and the resolution further enhanced by removal of albumin, perhaps by immunoadsorption. With the benefits of these improvements it seems certain that the resolution obtained by this method will bring to light new polymorphisms and possibly previously hidden variants of well characterised proteins.

Anderson and Anderson discuss the possible use of this technique in extensive human population surveys to assess mutation rates and possible increases that may be attributed to environmental pollutants. Surveys of this type may involve an unrealistic expenditure of energy and financial resources since it has been suggested that in order to detect a 50% increase in mutation rate about 6×10^6 obser-

vations would be required in each population sample (Neel, Tiffany & Anderson in *Chemical Mutagens* **3**, Plenum Press, 1973). However the use of 2-D electrophoresis and its application to tissues other than serum might mean that products of up to 300 loci could be analysed in each individual. This still means that 20,000 individuals would need to be tested in each population but the authors claim that the procedures can be automated, making such a screening programme a plausible proposition.

One of the intriguing aspects of this approach is that polypeptides of unknown as well as known function can be useful genetic markers. However, the pooling of data from a variety of tissues will necessitate some identification of polypeptides, as many proteins are common to different tissues.

The 2-D technique will clearly be appropriate for use in experimental mutagenesis and will allow the comparison of point mutation rate with more gross forms of genetic damage arising as a result of exposure to mutagens. The technique has already been used to detect and analyse the products of specific gene loci in mutants isolated using selective systems. Millman *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **73**, 4589; 1976) have analysed hypoxanthine phosphoribosyl transferase in thioguanine-resistant HeLa cells, and Steinberg *et al.* (*Cell* **10**, 381; 1977) have looked at cyclic AMP-dependent protein kinase in cyclic AMP-resistant clones of S49 mouse lymphoma cells. The full potential of the 2-D system as a way of screening a wide range of loci in parallel has yet to be realised. □

Biomathematics and cell kinetics

from N. MacDonald

WHILE battle rages in the correspondence columns of *Nature* over the claims of catastrophe theory to give a radical new way of looking at fundamental problems in biology, less ambitious activities continue in many fields of biomathematics, with occasional modest successes. Typically these are concerned with the precise formulation and elaboration of insights that stem from those intimately concerned with the empirical data, rather than with the imposition of formal structures *ex cathedra*.

Underlying many of the contributions at a recent conference in Paris* on 'Biomathematics and cell kinetics' was the work in the early 1970s by

F. J. Burns and I. F. Tannock (*Cell Tissue Kinetics* **3**, 321) and J. A. Smith and L. Martin (*Proc. natn. Acad. Sci. U.S.A.* **70**, 1263). These authors proposed that the life span of a eukaryotic cell, between two divisions, can be split into two qualitatively different phases A and B. The B phase consists of an ordered sequence of events, comprising the S, G₂ and M stages of the cell cycle as usually understood, together with possibly part of the G₁ stage. The A phase accounts for the considerable variation observed in the duration of the G₁ stage, and is taken to be a state in which the cell is not committed to progress round the cycle. Exit from the A phase to the B phase is taken to be random, and the consequences of this assumption, for the interpretation of labelling and synchronisation experiments, were explored. There is no direct way of looking at a cell and determining that it is in the A phase, so that this is a phenomenological model, not a direct identification as one has for the S and M stages.

At the Paris meeting, models in which exit from the A phase to the B phase is determined by attainment of a threshold were discussed by J. Hopper (La Trobe University) and S. Svetina (Ljubljana University), while the biochemical aspects of a rather different model of the cell cycle were described by F. A. M. Alberghina (Milan University). Specific applications of cell cycle models, including that of Smith and Martin, which were examined included circadian rhythms (M. Guiget, Paris University; N. R. Hartman, The Finsen Institute, Copenhagen) correlation between mother and daughter cells, or between sister cells (M. Rotenberg, University of California, La Jolla; P. D. M. MacDonald, McMaster University and Paris University) and the differentiation of stem cells. Stem cells have the choice of proliferation (staying in the cell cycle) or of differentiating, and this choice can be regarded as providing alternative exits from the A phase. The bone marrow stem cells attracted particular attention at the meeting. Here the differentiation can be into any one of several cell lineages which culminate in the various types of mature blood cell. The interaction of the control of stem cell proliferation and differentiation and the control of the subsequent proliferation and maturation processes in the different cell lineages provide many interesting problems.

The mathematical techniques used in these investigations can be stochastic, with simulation of the progress of typical members of a population of

*Held on February 27 and 28. Organised by A. J. Valleron and P. D. M. MacDonald of the Biostatistics Laboratory, Villejuif.

cells, or deterministic, as in differential equation models of the control of haemopoiesis. As in the case of simpler branching processes, it is possible to set down a version of a stochastic model of the cell cycle in a form using partial differential equations. Such a method, with appropriate cyclic boundary conditions, was described by S. P. Creekmore (Rand Corporation). Discrete deterministic models were discussed by A. Lindenmayer (Utrecht University).

Much of the detailed hard work in this field is concerned with attempts to extract unambiguous consistent information on the stages of the cell cycle from experiments which use a variety of techniques such as autoradiography, time lapse cinematography and synchronisation of cell splittings. A new technique that has provoked a burst of activity is flow cytometry, and several papers at the meeting were devoted to the interpretation of experiments of this kind.

A particularly interesting paper was that by T. B. L. Kirkwood (National Institute of Biological Standards and Control, Holly Hill) on a model of the commitment of human fibroblast cells to senescence. This relates to experiments which show that cultures of normal human diploid cells have a finite life span, measured in cell doublings, and that this life span falls with the age of the donor. The model is a stochastic one in which commitment to senescence takes place at cell division, committed cells then dividing normally for a given number of generations before the death of the resulting clone.

Since cell kinetics seems to have become defined as a different field from morphogenesis, relative little work was reported on spatial aspects of the growth of cell populations. However, R. Ransom (Freiburg University) discussed his simulations of the two-dimensional growth of clones in the imaginal disks of *Drosophila*, while W. Duchting (Siegen University) described the initial stages of an elaborate investigation of growth of a mixture of normal and tumour cells simulated as a square array. This is rather analogous to a probabilistic version of a Conway 'life game'.

Even without the inclusion of spatial aspects, some of the simulations presented at the meeting, such as those of granulopoiesis by Creekmore and by Duchting, were extremely elaborate and rich in parameters. Such simulations are not directly illuminating, and seek their ultimate justification, one supposes, in the view that if one can simulate normal and leukaemic granulopoiesis, for example, one can carry on to simulate the effects of the treatment of leukaemia by radiation or cytotoxic drugs. It was unfortunate that the only speaker scheduled to talk

on the optimisation of tumour therapy was unable to attend. However, E. Haus (Ramsay Medical Centre, St. Paul) and F. Halberg (University of Minnesota) kept the meeting from forgetting this practical aim of cell kinetics studies, and in particular its relation to circadian rhythms. It is to be hoped that at the next meeting in this series, which has been tentatively scheduled for La Jolla in 2 years' time, an appreciable fraction of the contributions will be concerned with applied cell kinetics. □

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Random fluctuations in high energy πp scattering

from Peter Litchfield

In a recent letter to *Physical Review Letters* (40, 429; 1978) a collaboration of physicists from Columbia University, Argonne National Laboratory and the University of Minnesota have reported the observation of random cross section fluctuations in high energy π^+p scattering. These fluctuations were predicted by Ericson and Mayer-Kuchuck (*A. Rev. Nucl. Sci.* 16, 183; 1963) and Frautschi (*Nuovo Cim.* 12A, 161; 1972) to arise from random diffractive phenomena due to interference from large numbers of superimposed resonance states. As such they give the first convincing evidence for the presence of the large numbers of resonances at high masses predicted by most models of the resonance spectrum, and also lend strong support to the concept of duality.

The experiment was carried out at the Argonne National Laboratory zero-gradient synchrotron. The elastic scattering of protons, π^+ and π^- mesons on protons was measured at large angles with high statistics and high angular and momentum resolution. A continuous beam momentum range from 2 to 9 GeV/c (centre of mass energies 2.1 to 4.3 GeV) was covered. Previous experiments have been limited to widely spaced beam momenta in this region. On examination of the π^+p and π^-p cross sections at fixed momentum transfer it was found that they could not be described by smooth functions of the centre-of-mass energy, but exhibited a number of fluctuations of up to a factor of two in cross section and with widths of the order of 100–200 MeV. The pp data showed no such effects.

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Random fluctuations due to interference effects have been known for many years in other branches of physics. In 1878 Exner (*Wiedemann Ann.* 4, 525; 1878) observed irregular patterns produced by a tiny frosted glass disk in coherent light and in astronomy similar effects give rise to diffraction-like intensity correlations over small time intervals when observing radio galaxies. In 1960 such effects were predicted in nuclear reactions by Ericson (*Phys. Rev. Lett.* 5, 430; 1960) and first demonstrated in 1962 by Colli *et al.* (*Phys. Lett.* 1, 120; 1962). It was quickly realised that similar conditions could occur in high energy scattering and indications of these effects were found in 1973 by F. H. Schmidt *et al.* (*Phys. Lett.* 45B, 157; 1973), but their measurements were limited by the very small range of centre-of-mass energy (beam momentum) covered. The present experiment with its high sensitivity to these effects and the confirmation that they occur in πp scattering where the formation of resonances is possible and not in pp where there are no intermediate resonances is strong evidence for the importance of this mechanism.

Almost all theories of the particle physics resonance spectrum, from explicit quark models to statistical models, predict a rapidly increasing density of resonances as a function of mass. This is borne out at low energies where resonances can be disentangled by specific partial wave analyses but it rapidly becomes technically impossible to perform detailed analyses as the mass of the formed resonances and the maximum angular momentum present increases. Crude attempts at analysis at high mass (A. W. Hendry, *Proc. of the Oxford Baryon Conf.*, 29; 1978) have shown that at least in the high orbital angular momenta resonances probably still occur. However, at high centre-of-mass energies, a seemingly entirely different reaction mechanism, the exchange of Regge poles, has long been recognised. This model is a generalisation of the particle exchange models that ascribe the forces between particles to the exchange of particles such as pions and ρ -mesons. Thus in the mid-1960s some confusion and controversy arose over when it was appropriate to use a resonance or a Regge pole description of data. The problem was resolved in the traditional manner of physics by the introduction of the concept of duality which states that the two concepts are equivalent. Thus the Regge exchange picture is thought to describe an average of the effects of all the resonances present and vice versa. Clearly, at low energies where there are few resonances the

average is a poor picture of the actual behaviour and it is more appropriate to describe the data by a resonance model, but, as the density of resonance increases, it is likely to be a better and better description and the simple Regge model with small numbers of exchanged particles is preferred.

If the fluctuations observed at Argonne are indeed due to the presence of large numbers of overlapping resonances then we have a striking confirmation of the concept of duality and also an indication that the predictions of explicit quark models for the superabundance of resonances at high masses may be correct. □

Magnetic bottles for cosmic rays

from a Correspondent

THE interaction of the solar magnetic field with the interplanetary medium and the Earth's magnetic field has been the subject of intense study in recent years by rocket and satellite probes. Solar activity in the form of solar flares gives rise to an increase in the flux of low energy cosmic ray particles in the vicinity of the Earth. Some solar flares are followed by intense 'magnetic storms' on Earth which cause a reduction in the cosmic ray intensity, referred to as Forbush decreases.

In 1966 cosmic ray detectors aboard the Pioneer 6 and 7 spacecraft found intense fluxes of ~ 10 MeV charged particles associated with disturbances in the interplanetary medium. The particle fluxes exhibited characteristic pulse profiles with a typical time scale of ~ 6 hours. At that time Rao *et al.* (*J. geophys. Res.* **72**, 4325; 1967) pointed out an almost complete correspondence between such events and the onset of Forbush decreases initiated by solar flares. Moreover the particle fluxes were strongly anisotropic with the direction of anisotropy showing sharp temporal variations. In addition following the decreasing phase of the Forbush decrease a 'bidirectional' anisotropy was frequently observed, the two directions of maximum flux being aligned parallel and antiparallel to the interplanetary magnetic field vector.

In 1960 Gold (*Astrophys. J. Suppl.* **4**, 406) proposed a model for the interplanetary magnetic field associated with solar activity based on magnetic field loops emerging from the Sun's surface and extending to large distances from the Sun (≥ 20 solar radii). Such a Gold 'bottle' model received strong support from Skylab observations in 1974 (Gosling & Roelof *Solar Phys.* **39**, 405).

However Rao *et al.* (*op. cit.*) argued that the Gold model could not explain the concurrent observation of both bidirectional and unidirectional fluxes of particles associated with Forbush decreases. They found their observations to be more consistent with the Parker 'blast wave' model in which a shock wave magnetic field disturbance moves out from the Sun. Indeed they argued that only the Parker model could adequately explain their observations.

Further experimental observations have now been reported by Palmer *et al.* (*J. geophys. Res.* **83**, 75; 1978) and these authors come to the opposite conclusion from Rao *et al.* Bidirectional anisotropies in low energy solar proton and electron events in the period 1967–73 have been investigated using data obtained from five satellites, Vela 5B, 6A and 6B and Explorers 34 and 41.

The Vela satellites are in circular orbit at 18 Earth radii distance, inclined at 60° to the plane of the ecliptic with an orbital period of 112 hours. Protons are detected at 10 different threshold energies in the energy range 0.3 to 45 MeV; electrons are detected at four different threshold energies in the range 28–214 keV and electrons of the same energies are detected after being scattered by a gold foil, thereby achieving good discrimination against contaminating proton fluxes. The Explorer satellites are in highly eccentric orbits covering the distance range 1 Earth radius to 35 Earth radii at inclinations of 67° and 87° with respect to the ecliptic plane. The Explorers detect protons in the energy range 0.7–125 MeV in five separate energy channels.

Sixteen observations of bidirectional distribution were detected in the 6 year period, including the observation of bidirectionality in three solar electron events. Each on average was of 9 hours duration. The average size of the region in which the bidirectional anisotropy existed was therefore ~ 0.13 AU based on a typical value of 600 km s^{-1} for the solar wind speed during disturbed conditions. Some of the observations were very short-lived. Two periods of electron bidirectional anisotropy lasted only 3 to 5 hours each. This implies that the bidirectional anisotropy was restricted to a region of small scale in one dimension. This does not fit with the picture of bidirectional anisotropy associated with large scale magnetic fields which lie between a blast wave and the Sun as suggested by Rao *et al.* (*op. cit.*). For agreement to occur the large scale fields must be structured on a much smaller scale.

As with the earlier data Palmer *et al.* find that the bidirectional anisotropy occurred at the minimum of the associated Forbush decrease in a majority

of the events. From this it can be argued that the bidirectionality occurred in a region immediately after the plasma field discontinuity which announced the onset of the Forbush decrease. The authors conclude that a Gold bottle model, or a magnetic regime containing large-scale loops behind an interplanetary shock wave, account best for the observations. This configuration gives rise to a bidirectional anisotropy when energetic particles injected from the Sun in one foot of the bottle are mirrored from the other foot. The Gold model accounts for the Forbush decrease minimum which correlates strongly with the bidirectional anisotropy in the solar cosmic rays. The short-lived period observed in energetic electrons indicates that the plasma fields near the front of the driver gas must be structured on a small scale in one dimension. The authors hypothesise that the driver gas consists of loops of field lines in 'shells' which are separated by tangential discontinuities. Thus the Gold bottle model for solar magnetic field disturbances seems to be gaining support. □



A hundred years ago

THOUGH the cultivation in India of the best quinine-yielding species of *Cinchona* (*C. officinalis*) has not proved a success, it is satisfactory to know that one species at least thrives most abundantly in the Sikkim plantations. From a paper read at the last meeting of the Pharmaceutical Society by Mr Wood, the Government Quinologist in India, it seems that out of a total of about three million trees, comprising four or five species of *Cinchona* it is estimated that there are as many as 2,500,000 belonging to the species *succirubra*. It is from this bark that the now well-known "Cinchona febrifuge" is prepared. This substance, according to many well known medical practitioners in India, possesses to so very nearly the same extent the anti-periodic properties of quinine that it may be safely substituted for the latter in the treatment of ordinary fevers and ague. 5,000 lbs. of this febrifuge, we are told, has already been made and issued, and it is now being made at the rate of 4,000 lbs. a year; the demand, however, is so rapidly overtaking this scale of production that a further extension will shortly be necessary. For use it appears in the form of a white powder, which, however, becomes in a short time of a pale buff tint. It does not agglutinate even in the Indian climate. It is freely soluble in weak acids and is readily taken up by lemon-juice, which constitutes a pleasant vehicle for its administration.

From *Nature* **17**, 21 March, 410; 1878.

review article

Direct measurement of molecular forces

B. V. Derjaguin, Y. I. Rabinovich & N. V. Churaev*

The current theory of the stability of colloids and liquid film rests on the direct measurements of the force of interaction between two surfaces separated by a thin film. The first measurements, in the 1950s provided the stimulus for Lifshitz's macroscopic theory of dispersion forces. So far, measurements of disjoining pressure fit well with the theoretical predictions; but there are still difficulties with measuring some of the components of that pressure.

THE surface forces whose radius of action is of the order of 10^{-5} – 10^{-6} cm play an especially important part in the phenomena of particle adhesion in colloid systems and in thin films of liquids. Surface forces set up an additional pressure, the disjoining pressure Π , in thin interlayers of liquid or gas between the surface containing those interlayers¹. Π is the difference between the pressure in a thin interlayer and that in the bulk mother phase, from which the interlayer has been formed. It defines the force of interaction (per unit area) of bodies that are separated by a thin plane parallel interlayer. A positive value of Π corresponds to repulsion, and a negative value to attraction.

Current theory of the stability of colloids and liquid films takes into account the simultaneous effect of several components of the disjoining pressure: Π_e , resulting from the overlapping of diffuse ionic layers on charged surfaces; Π_m , the forces of molecular attraction; Π_a , resulting from the overlapping diffuse adsorption layers of neutral molecules; Π_s resulting from the overlapping of boundary (solvate) layers of liquids, which have a modified structure; and a component resulting from the interaction of adsorbed layers of surfactants and polymers.

The development of the theory of these forces was preceded by experimental research on them. Thus, B. V. D. and Kuskov measured the thickness of the equilibrium wetting aqueous films that form when gas bubbles of various diameters are pressed against a glass plate immersed in the liquid. They were the first to plot the disjoining pressure isotherm, $\Pi(h)$, the dependence of the pressure Π in a thin film on its thickness h . The observed variation of the isotherm $\Pi(h)$ with valence and ionic concentration has given impetus to the development of the theory of the ionic-electrostatic component Π_e of the disjoining pressure²⁻⁵.

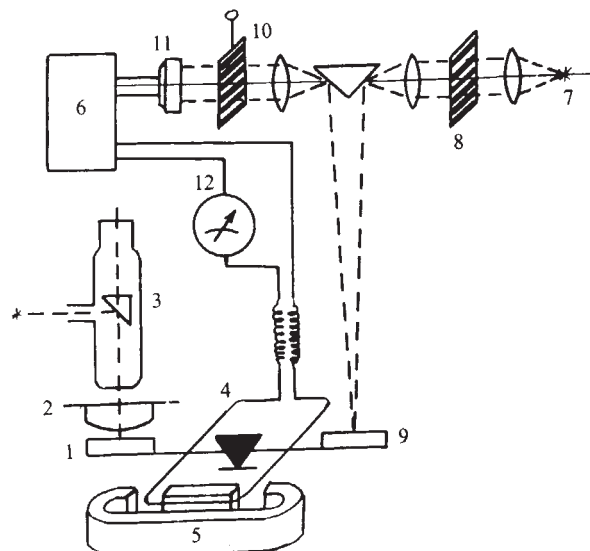
B. V. D. and Abrikosova⁶ were the first to measure the forces of molecular attraction between solids correctly. The main difficulty encountered in measuring the forces of molecular attraction of macroscopic bodies is that the forces decrease drastically as the distance between the solids increases. At large distances, they are extremely small, and so the accuracy of measurements is decreased. At small distances, the derivative $\partial\Pi_m/\partial h$ is large and positive and this makes it necessary to use dynamometers of high rigidity, which must be greater than $\partial\Pi_m/\partial h$. Complex methods are required to enhance the low sensitivity of such dynamometers.

In our experiments⁶⁻⁸, these difficulties have been overcome by using negative feedback for the simultaneous solution of two problems: the stabilisation of the distance h , and the measurement of the attraction force. Negative feedback allows the measured force to be automatically counterbalanced by maintaining a preset gap width h between the solids even in the range of large values of $\partial\Pi_m/\partial h$.

Figure 1 is a schematic diagram of the apparatus used in our research. The main part of the apparatus is a microbalance provided with negative feedback. The distance between the plate 1 and the lens 2 was calculated from the diameter of Newton's rings measured with the microscope 3.

The force of attraction of the solids 1 and 2 was compensated by the electric current flowing through a frame 4 which was rigidly attached to the beam of the microbalance. The balancing torque was created by the interaction of the electric current in the frame with the field of the magnet 5. The intensity of the electric current in the frame was preset by a follow-up device comprising a raster-type photorelay, a current amplifier 6, and a light source 7. The electric current was generated by the photoelement 11, when it was exposed to the light passing through rasters 8 and 10. The image of raster 8 was projected on raster 10. The quantity of light that passed through

Fig. 1 Schematic diagram illustrating the Derjaguin and Abrikosova apparatus for measuring molecular attraction forces in air and vacuum.



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raster 10, and consequently the illumination of the photoelement, depended on the overlapping of the images of raster 8 and raster 10. Thus the illumination varied as the mirror 9 turned.

A microammeter 12 was used to measure the intensity of the electric current balancing the attraction force. The relationship between the intensity of the current and the attraction force was determined on the basis of a calibration of the instrument. The distance h between the plate and the lens could be gradually changed by moving one of the rasters.

These first measurements^{6,7} conflicted with the London-Hamaker theory and provided the stimulus for the development, by Lifshitz, of the more widely-known macroscopic theory of dispersion forces^{9,10}. The basis of the London-Hamaker microscopic theory was the physical characteristics of separate molecules. The Lifshitz theory, by contrast, used the spectral characteristics of continuous media for calculating the attraction forces. The formulae of Lifshitz's theory relating to the distances at which the retardation effect is fully realised proved to be in good quantitative agreement with those first measurements of the molecular attraction (for $h = 0.07$ – $0.5 \mu\text{m}$). The retardation effect, as it is known, is connected with the finite velocity of propagation of electromagnetic fluctuations through the gap separating the solids.

Overbeek and Sparnaay¹¹ erroneously obtained values of the attraction forces. The formulae of Lifshitz's theory relating thousand times greater than the molecular attraction forces. Sparnaay tried to explain these overestimated values by modifying the London-Hamaker theory, but the actual cause of the error was the influence of residual electric charges¹².

As is known^{9,10}, in the case of electromagnetic retardation, the dependence of Π_m on the thickness h of a plane interlayer, has the form

$$-\Pi_m = B/h^4 \quad (1)$$

where B is a constant depending on the static permittivities of the interacting bodies.

B. V. D. has derived a formula¹³ relating the specific energy of the interaction of plane surfaces $u = \int_h^\infty \Pi_m dh$ to the force F of the attraction of non-planar bodies:

$$F = Cu(h) \quad (2)$$

where h is the shortest distance between the bodies and C is a geometrical factor. In particular, in the case of a lens and a plane plate $C = 2\pi r$ (where r is the radius of curvature of the lens); this yields the equation

$$F = 2\pi Br/3h^3 \quad (3)$$

For the case of cylinders crossed at right angles $C = 2\pi(r_1 r_2)^{1/2}$ where r_1 and r_2 are the radii of the cylinders; hence from equation (2) it follows that

$$F = 2\pi B(r_1 r_2)^{1/2}/3h^3 \quad (4)$$

These equations allow the values of B to be obtained from the experimental relation $F(h)$.

Derjaguin and Abrikosova⁹ obtained $B = (0.8 \div 1.4) \times 10^{-19}$ erg cm for quartz glass. Later, other workers^{14–20} obtained similar results using different methods of measurement of the forces and distances $B = (0.7 \div 1.2) \times 10^{-19}$ erg cm. For the interaction between a dielectric (quartz) and a metal (chromium), Derjaguin and Abrikosova obtained a larger value of $B = 2 \times 10^{-19}$, which was later corroborated by Rouweler¹⁹.

For a long time, the formulae of the Lifshitz theory could not be used because of the lack of data on the high-frequency absorption spectra. This difficulty is now gradually being eliminated, and the Lifshitz theory is becoming an appropriate basis for exact quantitative calculations. The work of Ninham and his colleagues²¹ has promoted further application of the theory in colloid science.

Tabor and Winterton²² were the first to investigate the region of considerably small distances between the solids. In this case, electromagnetic retardation can be neglected and the disjoining pressure isotherm has a different form:

$$-\Pi_m = A/6\pi h^3 \quad (5)$$

where A is Hamaker's constant.

For cylinders crossed at right angles, from equation (5) it follows that

$$F = A(r_1 r_2)^{1/2}/6h^2 \quad (6)$$

For small distances $h < 500 \text{ \AA}$, the polished surfaces that were used in the previous experiments proved to be insufficiently smooth because the height of their unevenness was commensurate with the width h of the gap. Tabor and Winterton therefore used thin sheets of atomically smooth mica glued on to glass cylinders with radii r of appropriately 1 cm. In these and further experiments²³ direct measurements were made of the attraction forces F between the cylinders crossed at right angles. These measurements allowed the values of both constants A and B to be obtained, and the transition region from one law of interaction (equation (1)) to the other (equation (5)) to be investigated. It has been shown that for mica, this transition takes place within the range of values of h from 120 to 500 $\text{\AA}$.

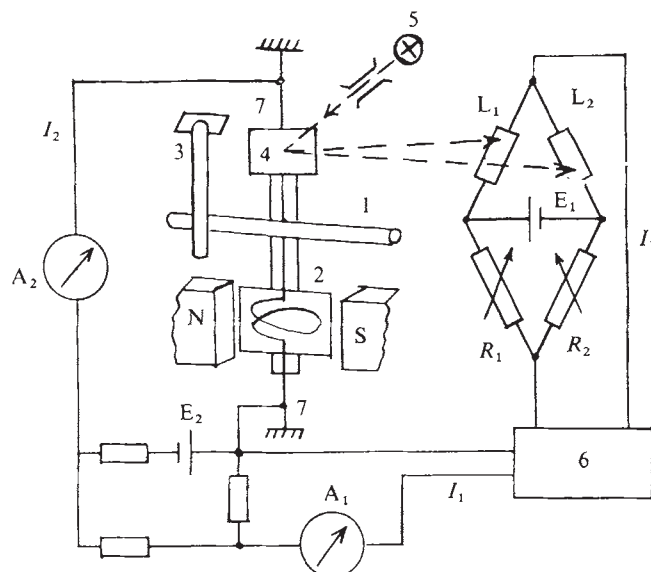
Unfortunately, the method developed by Tabor and co-workers for mica has not been extended to other substances. Moreover, measurements of the molecular forces have generally been limited to transparent bodies because the distance between them was usually measured by optical interferometry.

We have succeeded in measuring the forces of molecular attraction between metals, too, by applying a new technique²⁴.

The influence of surface roughness will decrease as the fraction ΔS of the surface area that produces the main contribution to the interaction of the bodies decreases (that is, as the radius of curvature decreases). We therefore used cylinders of small radius r (0.15–0.5 mm). It was necessary, however, to considerably improve the sensitivity of measurement of the forces (which are proportional to r , see equations (4) and (6)).

For this reason, a new method²⁴ was developed in which the shortest distance h between the crossed threads was not measured, but was preset by drawing apart the threads from

Fig. 2 Schematic diagram illustrating the Derjaguin, Rabinovich and Churaev apparatus for measuring molecular attraction forces in air.



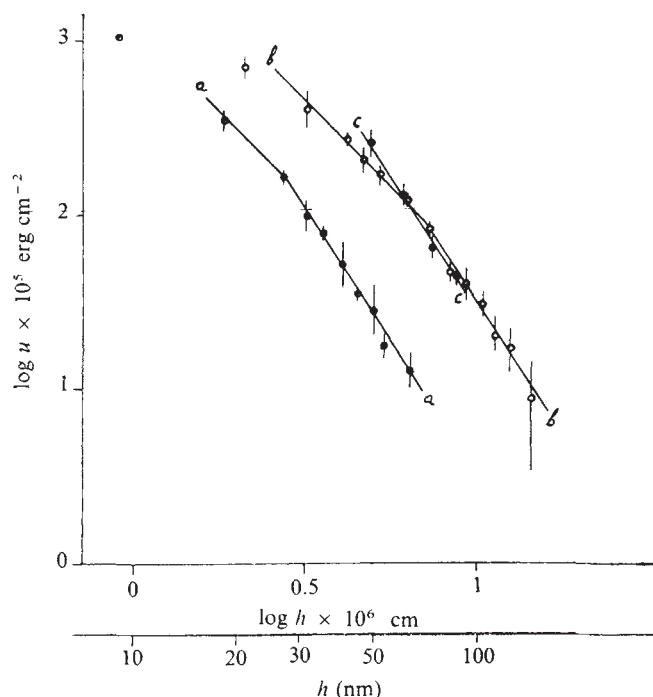


Fig. 3 Dependence of the energy of molecular attraction per unit area of plane-parallel surface on the gap h in the air. Quartz filaments: a ; platinum filaments: b ; gold filaments: c .

their position of mutual contact. A photoelectric circuit with negative feedback was used to perform three functions simultaneously: (1) to measure the force of attraction of the threads; (2) to guarantee the preset shift of the movable thread; (3) to ensure the stability of its position relative to the fixed thread in spite of the action of molecular forces. Such a method of measurement allows threads or filaments of any material to be used, including non-transparent (for example, metallic) ones.

The main part of the apparatus was a photoelectric galvanometer which was connected to the circuit represented in Fig. 2. One of the threads 1 was attached to the movable frame 2 of the galvanometer, which was placed in the magnetic field; the other thread 3 was fixed. The frame 2 of the galvanometer was suspended on thin bands (strips) 7. The light beam from the fixed source 5 was reflected from the mirror 4 which was attached to the frame 2 of the galvanometer. The light rays impinged on the photoresistances L_1 and L_2 , which formed a bridge circuit with the variable resistances R_1 and R_2 . In the absence of interaction forces, the frame 2 and the mirror 4 were set automatically in such a position that the current I_2 passing through the frame was zero. The current I_1 (of which current I_2 forms a part) in the diagonal of the bridge $R_1 R_2 L_1 L_2$ was then also zero. In the event of slight movement of the frame, the balance of the bridge was disturbed ($I_1 \neq 0$) owing to a variation in the illumination of the photoresistances L_1 and L_2 and the resulting current induced in the frame resets it to the equilibrium position.

By changing the resistance R_1 (or R_2) it was possible to vary the equilibrium position of the frame, that is, to move thread 1 a certain distance from thread 3. Thus at $I_1 = 0$ the values of R_1 and R_2 determined the value of h unambiguously. The accuracy with which the movement of the thread could be preset using this method was limited by the inherent oscillations of the frame, and amounted to about 10 Å.

When thread 1 was affected by the attraction force F , the galvanometer frame shifted from its equilibrium position, which had been preset at $F = 0$ by the resistances R_1 and R_2 . As a result, currents I_2 and I_1 were induced in the galvanometer frame and in the diagonal of the bridge, respectively. To determine the distance h between the threads using the values

of R_1 and R_2 , it was necessary to adjust I_1 to zero. This was achieved by switching in a variable current source E_2 . The current I_1 in the diagonal of the bridge was monitored with a microammeter A_1 placed after the amplifier 6.

The force of attraction of the threads was determined from the intensity of current I_2 , which was measured with a nanoammeter A_2 . By interaction with the magnetic field, the current I_2 flowing through the frame induces a torque moment balancing that of the attraction force F . The relation between the values of I_2 and F , as well as that between R_1 , R_2 and h , were found on the basis of an earlier calibration of the instrument.

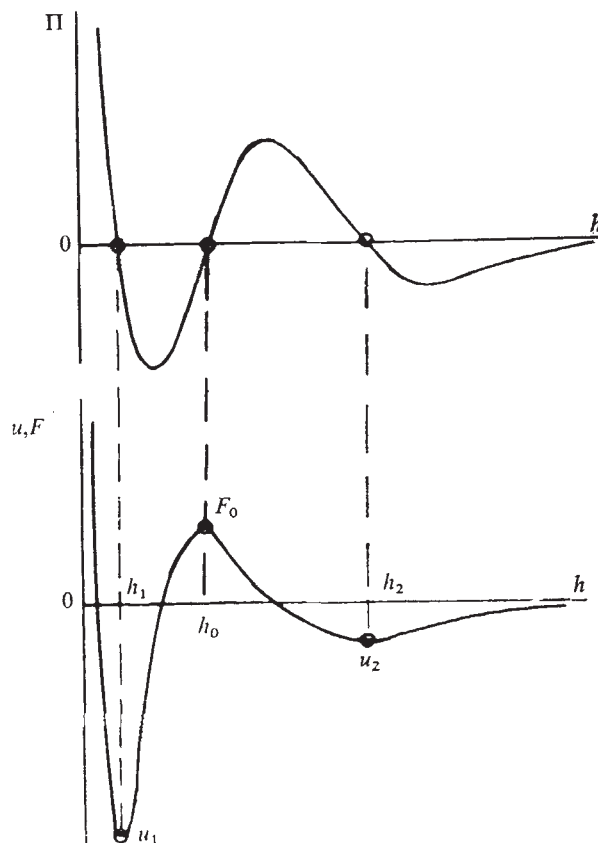
Figure 3 shows the dependence of $u = F/2\pi(r_1 r_2)^{1/2}$ on the distance h , calculated according to the experimental data. When plotted in logarithmic coordinates, the graph shows two linear sections that correspond to equations (4) and (6). We have succeeded in making reliable measurements for $h \geq 150$ Å in the case of atomically smooth quartz threads and for $h > 300$ –400 Å in the case of metal wires with a less smooth surface.

The constants A and B were calculated from the two linear sections of the curve in Fig. 3. For metals it was the first time that constants had been determined. The values of B for platinum and gold are both approximately equal to 1.0×10^{-18} erg cm. This value is close to the theoretical value of 1.3×10^{-18} erg cm, which is the same for all metals. For platinum, we also determined the constant A (2.0×10^{-12} erg).

We did not succeed in observing the transition to the non-retarded forces for gold because of the growing influence of the unevenness of the surface of the wires. In view of the absence of spectral characteristics, we have not yet determined the theoretical value of A for platinum. For gold $A = 4 \times 10^{-12}$ erg (ref. 25).

The value of $A = 5 \times 10^{-13}$ erg, which was obtained for quartz is close to that measured earlier by Wittmann and

Fig. 4 Dependences on the distance h of the disjoining pressure Π , the energy u of plane-parallel surfaces and the interaction force F of crossed cylinders in electrolyte solutions.



co-workers²⁰ ($A = 6 \times 10^{-13}$ erg). For quartz, the value of the constant B was found to be equal to 1×10^{-19} erg cm; this coincided with the value that was obtained in the experiment using the lens and plate, which we have already described.

For platinum, equation (4) is fulfilled for $h \geq 600$ – 700 Å, while for quartz it is fulfilled at considerably smaller distances $h \geq 270$ Å. This is because of the different values of the $A:B$ ratio for these materials.

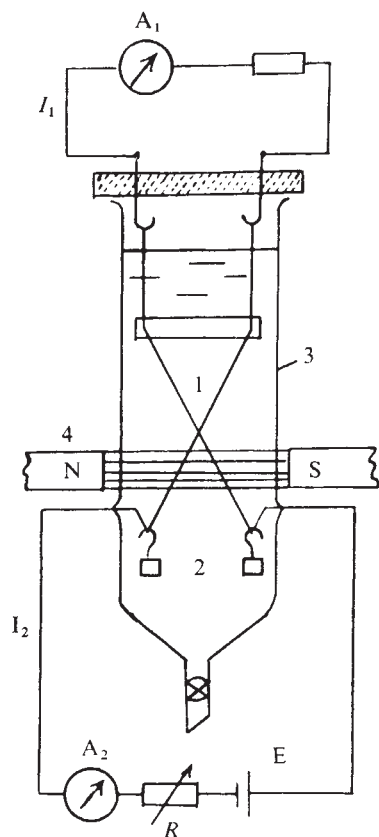


Fig. 5 Schematic diagram illustrating the apparatus for measuring the force barrier between crossed threads in an electrolyte solution.

So far, we have discussed the measurement of the forces of molecular attraction through gaseous interlayers. In some respects it is a more difficult task to make direct measurements of the molecular forces for bodies that are immersed in a liquid. Interpretation of the results obtained is made difficult by the simultaneous effect of several components of the disjoining pressure. Nevertheless, during recent years some progress has been achieved in this field too.

If several components of the disjoining pressure exert their effect simultaneously, the relation $\Pi(h)$ and $u(h) = F(h)/C$ usually have the form shown in Fig. 4. At large distances, the molecular attraction forces Π_m prevail over the electrostatic repulsion forces Π_e ; hence $\Pi = \Pi_m + \Pi_e < 0$. The relative contribution of the Π_e component increases as the solids approach each other. This force barrier must be overcome in order for the bodies to approach each other. Once the force barrier has been overcome, the molecular attraction forces again prevail, these forces being replaced by repulsion at a still shorter distance. The last effect may be ascribed either to the appearance of the Π_s and Π_a components of the disjoining pressure^{26,27} or to the influence of the force of Born's repulsion of the electronic shells of atoms.

The experiment involves the measurement of the interaction forces $F(h)$ between the crossed threads, and these forces are proportional to $u(h)$, as is evident from equation (2). Thus the force barrier F_0 for the threads corresponds to the maximum energy u_0 of interaction of the flat surfaces. The minima of the free energy correspond to the steady state of two plane parallel surfaces, the near one u_1 and the far one u_2 .

In order to determine the constant A , we used the extreme values of the function $u(h)$. Within the region between the energy barrier and the far minimum u_2 only two components of the disjoining pressure, Π_e and Π_m need to be taken into account. This approach is also used as the basis of the well-known DLVO theory^{2,5,28}. The calculation was made using $\Pi_e(h)$ isotherms, which are known theoretically, and equation (5) for $\Pi_m(h)$.

Direct measurement of the height of the force barrier was made for the first time in the 1960s^{29,30}. Thin ($r = 150 \mu\text{m}$) metal wires crossing at right angles were used to reduce the influence of viscosity. One of the threads was fixed and the other was made to approach it by being turned with an elastic torsion suspension. The height of the force barrier was determined from the maximum angle that the elastic suspension, must be turned through in order to bring the threads into 'contact' (the position $h = h_1$). Because the values h_1 were very small, the achievement of the 'contact' state was indicated by the appearance of electrical contact between the wires.

Using this method, we measured the force barrier F_0 as a function of the concentration and the valence of the electrolyte. Two values of the constant A were obtained on the basis of known values of the surface potential ψ_1 , which determines the value of Π_e . For gold-gold and platinum-platinum interactions through an interlayer of electrolyte we obtained³¹ $A \approx 4 \times 10^{-12}$ erg and $A \approx 2 \times 10^{-12}$ erg, respectively. The theoretical value $A = 3 \times 10^{-12}$ erg (ref. 25) for gold corresponds to the experimental value within the error limits. More recently, we have developed a simpler and more reliable method of measurement³². In this case, ponderomotive forces were used to overcome the power barrier. The schematic diagram of the apparatus is shown in Fig. 5. The platinum or gold wires 1 ($r = 25 \mu\text{m}$), in tensile stress due to the platinum weights 2, formed a bifilar suspension. The suspension was placed into a vessel 3 containing an electrolyte. The lower part of the crossed threads was placed within the field of a permanent magnet 4. The current I_2 from the source E was preset by varying the resistance R . On passing through the wires, it creates a force in the magnetic field, which twists the suspension and thus makes the threads approach each other until they contact. At the point of contact stepwise decreases in the current I_1 is noted on the milliammeter A_1 . The height of the force barrier can be determined from the maximum value of the current I_2 , measured with the milliammeter A_2 .

A distinctive feature of the arrangement is that a very small force is required to move the wires. We have succeeded in measuring the height of the barrier for gold threads at high concentrations of electrolytes, by this method.

Application of the method corroborated the results obtained in earlier experiments^{29,30} and allowed the distance between the threads (corresponding to the force barrier) to be approximately evaluated (Fig. 4). For KCl solutions, it was found to be equal to about 10–30 Å. It was also shown that there is no force barrier for the threads in the non-polar liquid CCl_4 , where only the attractive forces Π_m are effective.

Peschel *et al.*^{33,34} measured the forces of repulsion between a polished quartz lens and a polished quartz plate in various liquids. The dependence of Π on the temperature and concentration of the electrolyte was explained by the effect of the Π_e and Π_s components of the disjoining pressure.

Israelachvili^{35,36} has recently carried out measurements for curved mica plates in aqueous solutions, using the technique of crossed cylinders^{22,23}. The forces could be measured up to very small distances because of the molecular smoothness of the surface. With 1:1 electrolyte solutions a good agreement

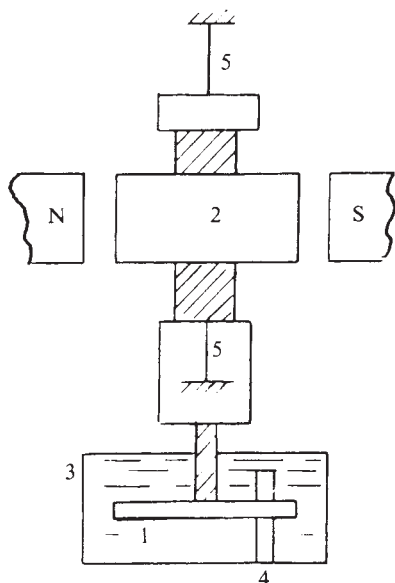


Fig. 6 Schematic diagram illustrating the apparatus for measuring the force barrier between crossed threads in an electrolyte solution.

with the DLVO theory was established. Deviations that might be ascribed to the effect of the Π_e component were observed only for $h \lesssim 30$ Å.

At $c = 0.1$ M when the electrostatic repulsion was suppressed, Israelachvili obtained a curve $F(h)$ with a minimum at a distance of about 70–80 Å. This relatively large distance for the position of the minimum implies that it is the secondary minimum h_2 (Fig. 4). Thus, using the DLVO theory (and a simplified expression for Π_e at $\chi h \gg 1$, where χ is the inverse Debye radius of diffuse ionic layers) we were able to determine the constant A from the condition that $dF/dh = 0$, by means of the values $u_2 = 4 \times 10^{-2}/2\pi$ erg cm $^{-2}$ and h_2 (from refs 35 and 36). The calculations we carried out yield, for mica in an electrolyte solution, $A = 1.8 \times 10^{-13}$ erg. Israelachvili *et al.*^{35,36} compared the experimental curve $F(h)$ with the theoretical curve for the region $h = 50$ –100 Å and obtained a value for A of 2.2×10^{-13} erg. Somewhat smaller theoretical values of $A = 1.1 \times 10^{-13}$ erg have been calculated for mica in water³⁷.

We have also applied our technique²⁴ to measuring the interaction forces for thin threads in liquids. In this case, the movable thread 1 was attached to the frame 2 of the galvanometer from below, so that the axes of rotation of the frame and of the thread coincided (Fig. 6). In this way we were able to eliminate the effect of surface tension forces of the liquid that was contained in the vessel 3. A fixed thread 4 was attached to the vessel 3 and was moved together with the vessel using a micrometric screw, so that the threads were close enough to contact on turning the frame. The measurements for glass threads 0.5 and 0.6 mm in radius were made in KCl solutions. The force sensitivity of the apparatus for measuring force was not less than 10^{-4} dyn. However, the point of contact ($h = 0$) from which the distances were measured was determined with an error of about 100 Å, because of insufficient rigidity of the clamps 5 (Fig. 6).

The following method was used to determine more precisely the point of contact ($h = 0$). As is well known²⁻⁵ for $\chi h \gg 1$ the logarithm of the electrostatic repulsion force F_e decreases linearly with distance. In this case

$$\ln F_e = K - \chi h \quad (7)$$

where K is a constant. This equation determines the slope of the linear dependences $\ln F(h)$ inasmuch as for each concentration it is easy to find $\chi \approx 3.3 \times 10^7$ c $^{-1}$ where c is the concen-

tration of the electrolyte.

The position $h = 0$ was determined more precisely by shifting the entire family of experimental points along the axis until the best fit with the theoretical dependence (7) was found in the region of large values of h .

Measurements were carried out for $h \leq 1500$ Å ($c = 3.5 \times 10^{-5}$ mol l $^{-1}$) and for $h \leq 800$ Å ($c = 4 \times 10^{-4}$ mol l $^{-1}$). The

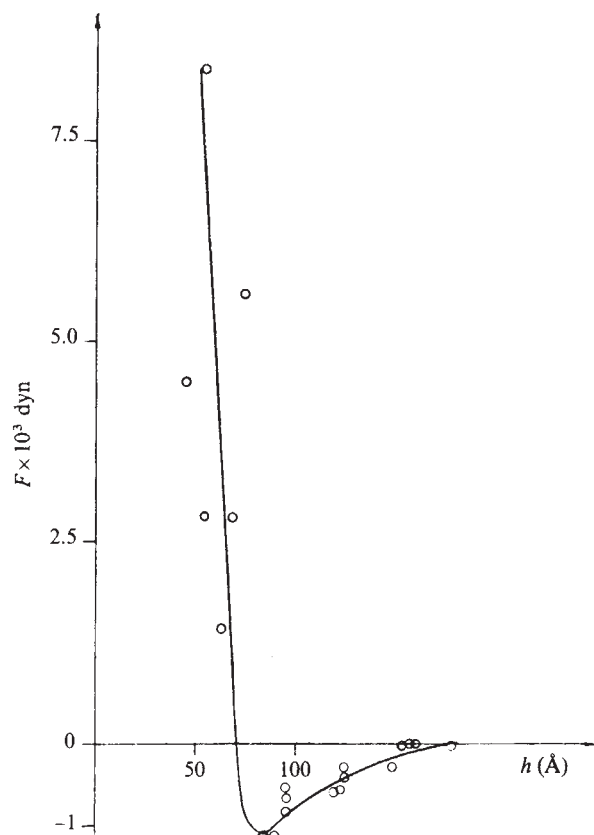


Fig. 7 Dependence on gap h of the interaction force F of crossed threads (1.0 and 1.2 mm in diameter) in KCl solution ($c = 0.1$ mol l $^{-1}$).

dependences of the repulsion forces on distance agrees well (up to about 100 Å) with the theory of the Π_e component. At these concentrations the molecular forces were only a small fraction of the interaction forces and could be disregarded.

At higher concentrations of electrolyte, the electrostatic forces decrease more rapidly with distance, thus allowing the effect Π_m of molecular attraction to be observed. Thus at $c = 0.1$ M we obtained the curve $F(h)$ with a minimum (Fig. 7). In this case, our earlier method of determining the contact position could not be applied. The origin of the h coordinate on the graph was therefore chosen arbitrarily. The only value that is exactly determinable is the minimum depth $F_2 = 1.1 \times 10^{-3}$ dyn.

For a high surface potential ψ_1 the calculated values of A are only slightly sensitive to its value; thus the constant A is determined solely by the value of F_2 . In a 0.1 M KCl solution, the experiments give for glass $A = 1.2 \times 10^{-13}$ erg. This value can be compared with the theoretical values for quartz $A = 0.6 \times 10^{-13}$ erg (ref. 37). Since glass has a higher refractive index than quartz, higher theoretical values of A are expected for glass. This should improve the agreement of experiment and theory.

Another method of measuring molecular forces is to use thin liquid films bounded on both sides by gas or another liquid. Thus, Sheludko and co-workers^{38,39} applied a dynamic method which involved measuring the rate at which films were thinning out under the effect of the molecular attraction of their surfaces. However, the method gives rather high values of the constants⁴⁰. This is probably due to the assumption that the surfaces of the film remain plane-parallel, as liquid flows from it. Lyklema and Mysels⁴¹ calculated the values of A using the equilibrium thickness of foamy films and a

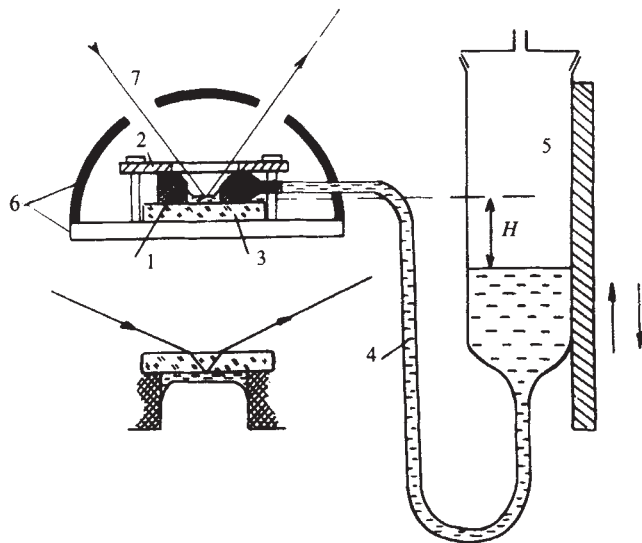


Fig. 8 Schematic diagram illustrating the set-up for measuring the disjoining pressure of wetting films of liquids.

number of assumptions relative to the form of the isotherm $\Pi_c(h)$. In the case of hydrocarbon films, where $\Pi_c = 0$, the values of A were determined from values of the contact angles between a thin film and the bulk liquid^{42,43}. The calculations were based on the known relation between the tension of the film and the $\Pi(h)$ isotherm.

In these experiments the constant A was found to be $(6 \div 7.5) \times 10^{-13}$ erg for water and the constant B was 1.9×10^{-19} erg cm for benzene, 4.9×10^{-19} erg cm for chlorobenzene and (4.6×10^{-19}) erg cm for aniline. Values of A range from about 2×10^{-14} to about 5×10^{-14} erg for various non-polar liquid films in water.

The nonsymmetrical case of the interaction of dissimilar solids has been studied less frequently. Measurements have been made only for quartz-chromium system in air at large distances^{6-8,19}. A good model of a non-symmetrical system is provided by wetting films. In this case, a solid and a gas interact through a liquid interlayer. Another different experimental technique is applied for wetting films, although the essential part of the research still involves obtaining the relation $\Pi(h)$ and interpreting them. However, instead of forces, the pressure in the liquid film is measured, while film thickness h is obtained by interferometry or ellipsometry.

Films of nonpolar liquids are convenient for measurements of molecular forces because the effect of the other components of the disjoining pressure can be neglected. Thus, for example, Blake⁴⁴ obtained values of $A \approx 5 \times 10^{-14}$ erg and $B \approx 4 \times 10^{-20}$ erg cm for decane films on sapphire; these are close to theoretical values.

Figure 8 is a schematic diagram of an experimental arrangement designed in our laboratory^{45,46}. The film is formed in an opening drilled in the porous filter 1 which is pressed by a Teflon cover 2 to the solid substrate 3. The disjoining pressure of the film is determined from the differences in the pressures

$\Pi = \rho g h$ where ρ is the density of the liquid and g is the gravitational acceleration. Beams of polarised light from an ellipsometer pass through the window 7 in the wall of the chamber.

Using this method, we obtained values for A of 5×10^{-14} erg for tetradecane on mica, 5.3×10^{-14} erg for glass, 10^{-13} erg for sapphire and 7×10^{-13} erg for steel. The value of the constant B for tetradecane on steel (1.6×10^{-19} erg cm) agrees well with the theoretical value $B = 1.75 \times 10^{-19}$ erg cm⁴⁰. We found a noticeable influence of the unevenness of the polished surface on the form of the $\Pi(h)$ isotherm. For the atomically smooth surface of mica, the isotherms exactly follow the theoretical equations (5) within the entire range of film thickness examined (60–200 Å).

This brief review mainly covers the results of our own experiments and those of our co-workers. In general, much success has been attained in this field of surface science.

Theoretical and experimental research into the Π_c and Π_m components of the disjoining pressure do not reveal serious discrepancies. Other components of the disjoining pressure have been studied but to a smaller extent. The difficulties that arise are ascribed first of all to the very high sensitivity of the Π_c and Π_a components to the composition of the liquid and to the real state of the surface. Moreover, it is necessary to measure forces that change drastically with distance. Thus the experiments that have now been successfully applied to the examination of the electrostatic and the molecular forces are made more complicated and difficult.

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X-ray crystal structure analysis of plastocyanin at 2.7 Å resolution

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The three-dimensional structure of plastocyanin, a 'blue' or 'Type 1' copper-protein, has been determined at a resolution of 2.7 Å. The copper atom has a highly distorted tetrahedral coordination geometry. It is coordinated by a cysteine thiol group, a methionine thioether group, and two histidine imidazole groups.

THE nature of the copper centres in 'blue' or 'Type 1' copper-containing proteins is one of the intriguing questions in biological inorganic chemistry. Such centres possess a combination of properties which has not yet been mimicked in any 'model' complexes: an absorption band near 600 nm with an extinction coefficient one to two orders of magnitude greater than that of most other Cu(II) complexes, an electron paramagnetic resonance (EPR) spectrum with a very small hyperfine splitting constant $A_{\parallel}$, and a redox potential appreciably higher than that of the Cu(II)/Cu(I) couple in aqueous solution¹. The high redox potentials of the 'blue' Cu-proteins imply that the coordination of the Cu atoms is such as to destabilise Cu(II) with respect to Cu(I). An electron-transfer function has been established for two 'blue' Cu-proteins, plastocyanin and azurin. 'Type 1' Cu centres may occur in proteins alone or in association with 'Type 2' and 'Type 3' centres which have different, but also distinctive, properties¹.

Plastocyanin is a protein with a single polypeptide chain, a molecular weight of 10,500, and a single Type 1 Cu atom. Its electron-transfer function is essential in photosynthesis². The amino acid sequences of plastocyanin from several algal and plant sources have been published³. Plastocyanin from poplar leaves (*Populus nigra* var. *italica*) was crystallised in a form suitable for X-ray diffraction measurements following unsuccessful crystallisation experiments with plastocyanins of 13 other plant species⁴. Plastocyanin from pea leaves has been crystallised in another laboratory⁵. We report here the structure of the oxidised (Cu(II)) form of poplar plastocyanin at a resolution of 2.7 Å. A refinement of the structure using diffraction data to a limit of 1.8 Å, and a structure analysis of reduced (Cu(I)) poplar plastocyanin are in progress.

Structure analysis

Cu(II)-plastocyanin from poplar leaves was isolated, purified, and crystallised by vapour diffusion against 2.6 M (NH₄)₂SO₄ at pH 6.0 (0.1 M sodium phosphate buffer). The intensely blue crystals were orthorhombic, space-group $P2_12_12_1$, with $a = 29.61$ Å, $b = 46.86$ Å, $c = 57.60$ Å and one molecule per asymmetric unit. If the partial specific volume of the protein was assumed to be

$0.74 \text{ cm}^3 \text{ g}^{-1}$, it followed that 36% of the volume of the crystals was occupied by solvent⁴.

The X-ray intensity data were measured on an Enraf-Nonius CAD-4/F diffractometer (CuK α radiation, ω -scans, background measurements at the extremes of each scan). A single crystal was used to record each of the native and heavy atom derivative data sets. The data were corrected for decomposition (less than 10% in each set) and absorption. Two derivatives were prepared by successively exchanging the phosphate buffer in which the crystals were grown against three fresh solutions of sodium acetate buffer at pH 6.0, and then soaking the crystals in a fourth buffer solution containing uranyl acetate (2 mM) for 23 h and 10 d, respectively. A third derivative was prepared by soaking crystals in mercury(II) acetate (50 mM) in phosphate buffer for 23 h. This procedure caused the crystals to become colourless. Structure analysis showed that Hg(II) had replaced Cu(II) at the metal-binding site of the protein. A fourth derivative was obtained with tetrachloroplatinate(II) in phosphate buffer. The diffraction data recorded from this derivative were on the average only half as intense as those of the native protein, and were not used in the phase-determining calculations. The major heavy-atom sites of the UO₂²⁺ and Hg(II) derivatives were located by difference and anomalous Patterson syntheses. The minor sites in these two derivatives, and several weakly occupied sites in the PtCl₄²⁻ derivative, were found by difference Fourier syntheses. The heavy-atom parameters were refined by the least-squares method of Dickerson *et al.*⁶ (Table 1). The overall figure of merit for the 2,241 reflections to 2.7 Å was 0.83. A best Fourier synthesis⁷ was computed. The contribution of anomalous scattering was included in the phase calculations⁸. A skeletal model was fitted to the electron density map using Watson-Kendrew components in a modified Richards optical comparator^{9,10}.

The boundary of the molecule was clearly defined in the electron density map. Regions occupied by solvent were relatively unstructured. Connected electron density accounted for all 99 residues of the peptide backbone. Preliminary sequence data for the poplar protein had been made available by R. Ambler. Most of the expected side chains could be accommodated in suitable electron density. The map was weakest in the region of residues 55–59. The correctness of the model in this vicinity was confirmed when it was noted that the side chain of residue 57 (Met) had been placed at about 2 Å from the major Pt(II) site in the PtCl₄²⁻ derivative.

The highest peak in the map was assigned to the Cu atom. This was a spherical peak joined by continuous density to four points on the peptide backbone. The position of the peak coincided with the site of the Hg atom in the HgAc₂ derivative. The possibility that the electron density in the vicinity of the Cu peak had been perturbed by the substitution of Hg(II) for Cu(II) was investigated by calculating a map with phases derived from the UO₂²⁺ derivatives alone. This map, although of generally lower clarity than the three-derivative map, revealed substantially the same Cu peak and environment.

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Table 1 Heavy atom parameters

Derivative	<i>d</i>	<i>Z</i>	<i>x</i>	<i>y</i>	<i>z</i>	<i>B</i>	<i>N</i>	$\langle f_h \rangle$	$\langle E \rangle$	<i>R</i>
Uranyl acetate (23 h)	2.7	48.5	0.015	0.363	0.544	22.9	2,207	57.4	25.0	0.15
		12.7	0.816	0.392	0.304	25.0*				
Uranyl acetate (10 d)	3.0†	79.1	0.018	0.361	0.544	20.3	1,567	116.1	45.4	0.21
		46.9	0.819	0.393	0.304	26.8				
				(+ 5 additional sites of low occupancy)						
Mercury acetate (23 h)	2.7	43.9‡	0.237	0.747	0.327	17.0	2,241	56.4	22.7	0.13

d, Resolution limit of data (Å); *Z*, relative occupancy of heavy atom site in electrons. (The scale factor of the native data was determined by a Wilson plot); *x*, *y*, *z*, fractional coordinates; *B*, isotropic temperature factor (Å²); *N*, no. of reflections included for this derivative; $\langle f_h \rangle$, mean heavy atom scattering (electrons); $\langle E \rangle$ is the root mean square lack of closure error (electrons); reliability index, $R = [\sum (F_{ph} - F_{phe})^2 / (\sum F_{ph}^2)]^{1/2}$, where F_{ph} = heavy atom derivative structure factor amplitude and F_{phe} = calculated heavy atom structure factor amplitude.

*Parameter held constant in refinement.

†Data for this derivative were restricted to lower resolution on account of significant (up to 1%) changes in the cell dimensions.

‡The Hg atom parameters were refined with (Hg²⁺-Cu²⁺) scattering factors.

Description of the structure

The shape of the plastocyanin molecule resembles a slightly flattened cylinder with dimensions 40 × 32 × 28 Å (Figs 1 and 2). The walls of the cylinder are formed by eight strands of polypeptide chain roughly parallel to the cylinder axis. At this stage of the structure analysis many of the hydrogen-bonded contacts between adjacent strands have yet to be defined with certainty. However, substantial portions of the polypeptide chain in seven of the eight strands are seen to be in the correct configuration for β -pleated sheet. The remaining strand has an irregular conformation apart from a single turn of α helix between residues 51 and 54. The β -sheet topology of the molecule (Fig. 3) is not identical with the β topology of any of the proteins listed by Richardson *et*

al.^{12,13}. Note that this view of β topology is highly idealised—structural irregularities in the β strands, and differences between the lengths of corresponding β strands in different structures, are ignored.

The core of the molecule is hydrophobic and notably aromatic because of clustering of six of the seven Phe residues. Polar side chains are distributed unevenly on the exterior of the molecule, so that the net negative charge at physiological pH (*pI* ~ 4) is located significantly more on one side than the other. There is an elongated negative region extending from the carboxylate groups of residues 42–44 to those of residues 59–61. Five of these six residues are conserved in all higher plant plastocyanins³. Another distinctive feature of the structure is a hydrophobic patch formed by several residues (10, 12, 35, 36, 89–91) on the loops joining strands 1–2, 3–4 and 7–8 at one end of the molecule. This hydrophobic patch surrounds an opening beneath which the Cu site is located.

The copper binding site

The Cu binding site is embedded between the ends of strands 3, 7 and 8 of the protein backbone. The Cu is coordinated by the sulphur atoms of Cys 84 and Met 92, and by the δ nitrogen atoms of the imidazole groups of His 37 and His 87 (Fig. 4). Thus three of the four donor atoms belong to residues in the sequence CysXXHisXXXMet which forms the tight loop between strands 7 and 8. In one direction, access to the Cu atom from the solvent is blocked only by the imidazole group of His 87. The approach of solvent molecules to the Cu atom from this direction is limited to about 6 Å. At this stage the coordination geometry seems to be irregular, with bond angles deviating by as much as 50° from the values for a tetrahedron. Better definition of the coordination geometry will be available when the structure is refined.

Biological implications

Presumably it was the accessibility of two oxidation states which led to the selection of copper as the active centre of an electron-transfer protein. If this is so, then evolutionary pressures will have tended to optimise the copper environment for this purpose. In the terms of the 'simple hard-soft acid-base' (HSAB) theory^{14,15}, the categories of Cu(II), Cu(I) and the ligand groups are

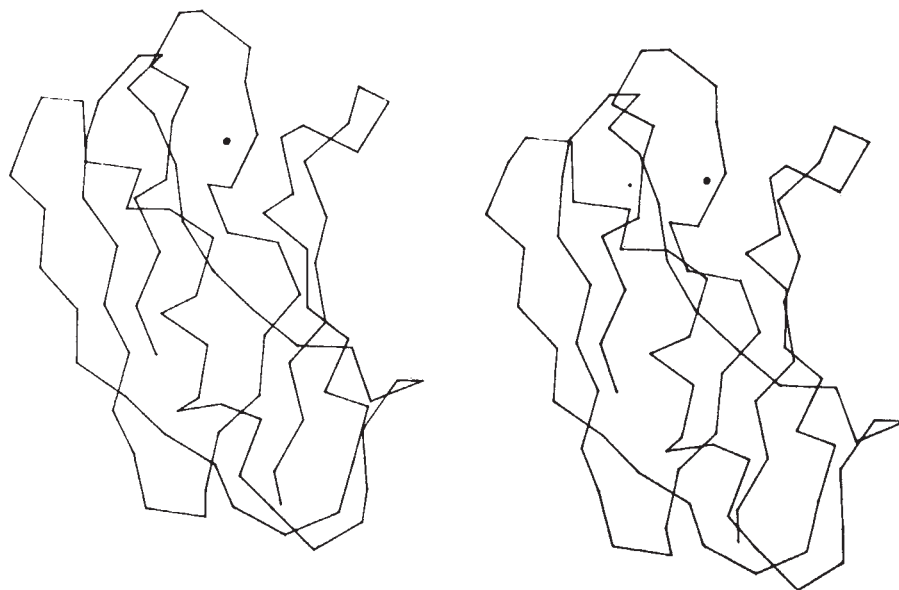
	'Hard'	'Intermediate'	'Soft'
Acid	—	Cu(II)	Cu(I)
Base	—	Imidazole	RS ⁻ , R ₂ S

For each of the two oxidation states of the Cu atom, the ligands in plastocyanin include two groups which are in the same HSAB category and two which are in the adjacent category. The ligand groups thus represent a compromise between the requirements of copper in its two oxidation states, so that reduction of the Cu(II) protein can proceed without changes in the coordination. It remains to be proved by means of a structure analysis of Cu(I)-plastocyanin that this is the case. Incidentally, such a

Fig. 1 The polypeptide chain in poplar plastocyanin. The circles represent α -carbon positions derived by applying the model-fitting procedure of Diamond¹¹ to atomic coordinates measured on the Watson-Kendrew model. Every tenth residue and the four copper-binding residues are numbered following the scheme of Boulter *et al.*³ for higher plant plastocyanin sequences. The letters N and C denote the NH₂-terminal and COO-terminal residues, respectively. The approximate directions of the Cu-ligand bonds are indicated.



Fig. 2 Stereo drawing of the polypeptide chain of plastocyanin, in the same orientation as in Fig. 1. The dot represents the position of the copper atom.



combination of ligand groups is advantageous for 'outer-sphere' electron-transfer mechanisms (see below) which require the first coordination sphere to remain intact.

The coordination geometry is distorted from the normal tetragonal or planar geometry found in Cu(II) complexes of low molecular weight. The protein molecule can compensate for localised strain through the free energy gained by the formation of hydrogen bonds, ionic interactions and 'hydrophobic bonds' elsewhere in the molecule: while the free energy is minimised for the molecule as a whole, there may well be pockets (such as the Cu centre in plastocyanin) where this is not the case. Plastocyanin provides a good example of 'entasis'¹⁷. The effect of the distortion of the Cu geometry is to reduce the activation energy for electron transfer and to enhance the redox potential¹⁶. A high redox potential is probably required because plastocyanin is part of a photosynthetic electron-transfer sequence in which its redox partners have high redox potentials.

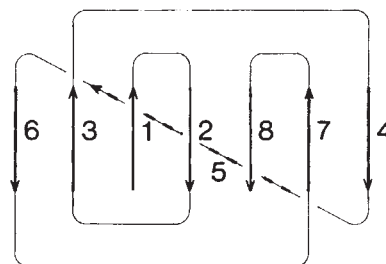
How are electrons transferred to and from the Cu centre? One of the two currently favoured hypotheses for electron transfer between metalloproteins¹⁸ extends to metalloproteins the concept of 'outer-sphere' interaction which is applied to many electron-transfer reactions between inorganic complexes. In an outer-sphere mechanism, the metal-ligand bonds in both the reacting species are unbroken. The transition state involves overlap between orbitals on the ligands in the two complexes. For electron-transfer between two metalloproteins, the outer-sphere hypothesis requires the two proteins to be in contact at a point to which an electron can be delocalised from the metal atom of the reducing protein, and from which it can be delocalised to the metal atom of the acceptor protein. A special case of delocalisation is represented by conjugated pathways (for example, that between the Fe atom and the haem edge in cytochrome *c*)¹⁹. In plastocyanin this type of electron transfer could occur by way of the coordinated imidazole ring of His 87. As noted previously, this ring is all that separates the Cu atom from the surrounding medium at one end of the plastocyanin molecule. Further, the hydrophobic residues which surround the opening to the Cu binding site are highly conserved in plastocyanins³. The absence of negatively charged side chains from the vicinity of His 87 should also make this region of the molecule attractive for interactions with negatively charged inorganic reductants and oxidants such as $\text{Fe}(\text{CN})_6^{4-}$ and $\text{Fe}(\text{CN})_6^{3-}$. Electron transfer between plastocyanin and such complexes is considered to proceed by outer-sphere mechanisms²⁰.

A second hypothesis explains electron transfer to and from a protein metal centre in terms of quantum-mechanical tunnelling along 'hydrophobic channels' (channels lined with nonpolar side chains). It is possible to identify several such channels in plastocyanin since most of the side chains in the interior of the

molecule, with the exception of those which bind the Cu atom, are hydrophobic. There is one hydrophobic channel which is particularly interesting because it starts at the periphery of the molecule in the region which has been described as carrying predominantly negative charges. This channel originates near Tyr 83 and is lined by the side chains of Phe 82, Val 93, Gly 94 and Phe 14. In plant plastocyanins all these residues are invariant³. There are minor variations in algal plastocyanins. These observations suggest that this part of the plastocyanin molecule has a functional importance. In addition, as will be discussed below, residues corresponding to Phe 82 and Tyr 83 are conserved as Phe-Phe or Tyr-Phe in all azurins.

The observation that there are at least two plausible pathways for electron transfer in plastocyanin is consistent with the further hypothesis that direction is important in the control of biological electron transfer¹⁸. For plastocyanin this hypothesis means that one path is required for an electron from the reductant (probably cytochrome *f*) to reach the Cu, and a second path is needed to transfer the electron to the oxidant (probably P700). The distinctly hydrophilic and hydrophobic patches on the exterior of the molecule may then provide specific sites of interaction between plastocyanin and its two redox partners. Specificity is implied by the fact that the *in vitro* rate of reduction of parsley plastocyanin by parsley cytochrome *f* is 30 times higher than the rate for any other cytochrome examined²¹, and three orders of magnitude higher than the rate of reduction by inorganic reagents²⁰. The need for specific sites of interaction may exist even if the molecules tumble freely in solution. On the other hand, if plastocyanin and the components of photosystem I are located in the thylakoid membrane², then the existence of recognition sites may be necessary to ensure that the plastocyanin molecule is appropriately oriented with respect to groups in the membrane as well as (or instead of) the redox partners.

Fig. 3 Schematic representation of the topology of the plastocyanin molecule, using the notation of ref. 13. The cylindrical barrel formed by the eight strands of the polypeptide backbone (numbered 1-8) is cut between strands 5 and 6, and is laid flat on the page with the outer surface uppermost. Portions of strands 1-4 and 6-8 are linked into β sheet. The irregular strand 5 completes the cylinder by joining strands 4 and 6.



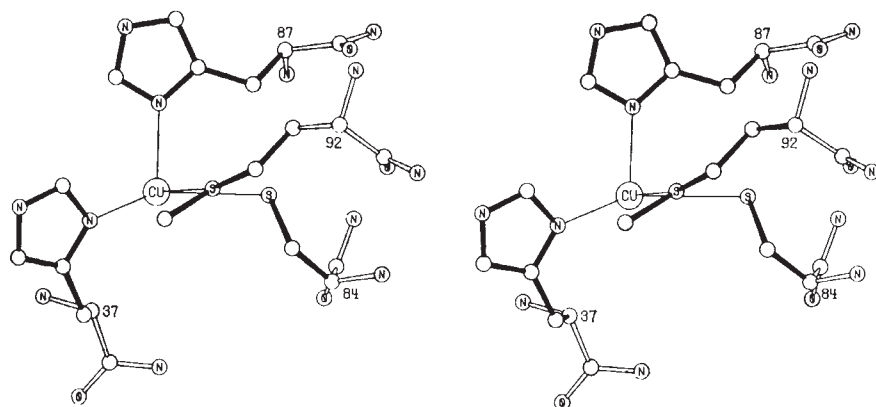


Fig. 4 Stereo drawing of the copper-binding site in plastocyanin, showing the four ligand residues (His 37, Cys 84, His 87 and Met 92).

Relationship of poplar plastocyanin to other 'Type 1' copper proteins

The fact that poplar plastocyanin was chosen for structure analysis by trial-and-error does not reduce the generality of our conclusions. The high-field proton NMR spectra of a series of plant plastocyanins prove that the environment of the Cu atom is highly conserved²². Of the 99 amino acid residues in poplar plastocyanin, 28 are invariant in all plastocyanins³. The invariant residues include the four Cu ligands, 5 of the 6 residues which define the elongated negative region mentioned above, and 6 of the 7 residues which make up the hydrophobic patch at the end of the molecule near the Cu site. The six additional residues found in the sequence of an algal plastocyanin³ occur at the NH₂- and COO-termini and at three positions on the large loop connecting strands 6 and 7 where they are unlikely to cause a major change in the overall structure. In another algal plastocyanin, residues 57 and 58 are missing³. Deletion of these residues from the poplar plastocyanin structure would merely straighten a small kink in the irregular strand of the protein backbone. Thus, there is good reason to believe that other plastocyanins have essentially the same structure as poplar plastocyanin.

Of the other single-Cu Type 1 proteins¹, stellacyanin has a Cu-binding site which must differ from that in plastocyanin with respect to at least one coordination position, as stellacyanin contains no Met residues²³. In view of the very high E^0 values of Cu(II) complexes with cyclic thioether ligands²⁴ it is interesting that stellacyanin, which has no thioether side chain, has the lowest redox potential (0.184 V) (ref. 25) of any Type 1 Cu-protein. The absence of a Met in stellacyanin is, however, only one aspect of a marked lack of sequence homology between stellacyanin and plastocyanin in the regions of their respective single Cys resi-

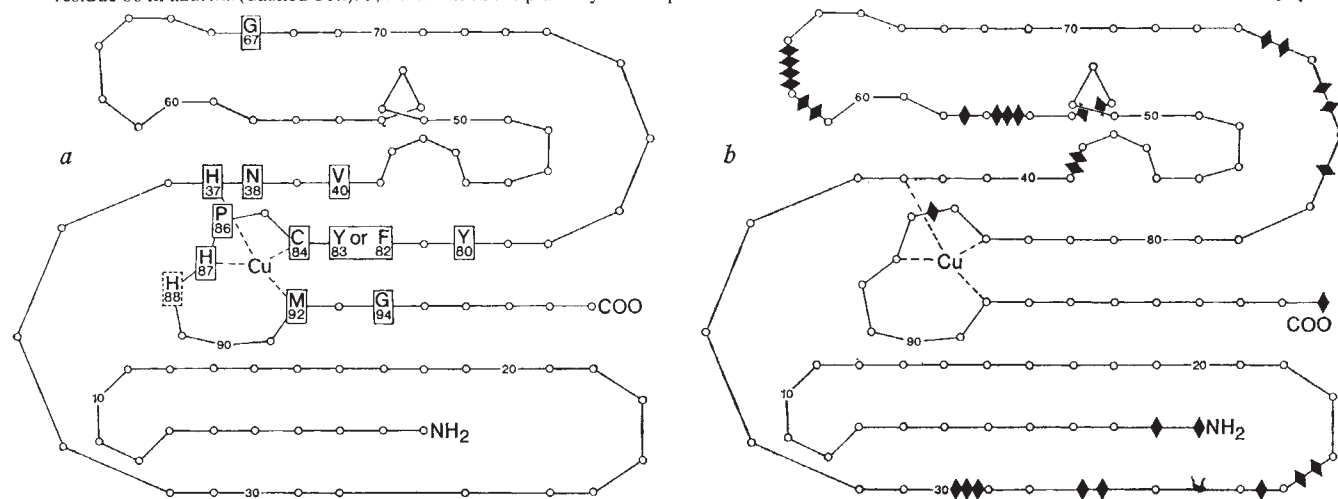
dues²⁶. Further evidence that the Cu-sites in stellacyanin and plastocyanin are significantly different is provided by EPR²³ and resonance Raman²⁷ spectra.

The Type 1 Cu-protein which is apparently most closely related to plastocyanin is azurin, a bacterial electron-transfer agent. Sequences of the approximately 130 amino acid residues have been determined for azurins from several sources. Structural homologies between the azurins and plastocyanins have been noted^{3,28-30}. When provision is made for differences between the polypeptide chain lengths of the azurins and the plastocyanins, it is possible to align the amino acid sequences of the two families of proteins in a way which brings a significant number of identical or similar residues into correspondence. This has led to a proposal that the two types of protein have diverged from a common ancestor³¹. We now examine the possibility that the plastocyanins and azurins are related not only at the level of primary structure but also at the level of tertiary structure.

In Fig. 5 the topological diagram for plastocyanin from Fig. 3 is rearranged by making the cut in the cylinder between polypeptide strands 3 and 6 instead of 5 and 6. The irregular strand (strand 5) is given equal weight to the other strands instead of being drawn merely as a connecting segment. This representation brings together in two dimensions the portions of the structure which are close to the Cu site. It also emphasises the somewhat detached nature of the large lobe formed in plastocyanin by strands 5 and 6 between residues 50 and 74.

We have marked in Fig. 5a those residues which are conserved in all plastocyanins and azurins³¹. In plastocyanins, all except one of these residues are now known to be either Cu ligands or to be in parts of the sequence which fall close to the Cu site. The positions where additional residues occur in the azurins³¹ (Fig. 5b) are

Fig. 5 Topological diagram for plastocyanin, re-arranged from Fig. 3 by making the cut in the cylindrical barrel between strands 3 and 6 of the polypeptide chain. Some of the major departures from regular structure are indicated schematically, but no inferences about hydrogen-bonding between adjacent strands should be drawn from the alignment of residues in other parts of the diagram. Residues are numbered as in Fig. 1 to correspond with the amino acid sequences of higher plant plastocyanins³. a, Rectangular boxes enclose residues which, in Ryden and Lundgren's correlation of the plastocyanin and azurin sequences³¹, are invariant in all plastocyanins and all azurins. An exception occurs at residue 87, where we show a conserved His (solid box). Ryden and Lundgren³¹ place the conserved His at residue 87 in plastocyanins and at a position corresponding to residue 88 in azurins (dashed box). b, Positions in the plastocyanin sequence where additional residues occur in the azurins³¹ are denoted by ♦.



nearly all remote from the Cu site in the plastocyanin structure, and occur in parts of the sequence where they can be accommodated without necessarily causing serious disturbance to the entire structure. When taken together, Fig. 5a and b suggest that the azurin and plastocyanin families have similar Cu sites in keeping with their similar biological roles as electron-transfer proteins, and that the molecular structures are also basically similar.

Chemical implications

The discovery of an irregular coordination geometry for the Cu atom in plastocyanin is not surprising. A tetrahedral distortion from square-planar coordination was originally predicted on theoretical grounds to explain the unusual features of 'Type I' Cu-protein EPR spectra^{32,33}. The very small hyperfine splittings $A_{||}$ could be reproduced in the EPR spectra of model Cu(II) complexes when the coordination geometry was tetrahedral³⁴ or distorted from square-planar towards tetrahedral³⁵. It was also noted that the redox potentials of Cu(II) complexes tend to move to more positive values in response to distortions from square-planar (or tetragonal) coordination³⁶⁻³⁸. The electronic, circular dichroism (CD) and magnetic circular dichroism spectra of 'Type I' Cu-proteins and derivatives in which Cu(II) was replaced by Co(II) were shown to be consistent with Cu sites having low, probably distorted tetrahedral, symmetries³⁹⁻⁴¹.

The location of the Cu site in plastocyanin was stated to be 'relatively inaccessible to solvent' on the basis of proton relaxation measurements⁴². Similar results were reported for azurin and umecyanin⁴³. The minimum Cu-proton distance calculated from proton relaxation measurements on azurin⁴⁴ is in agreement with our estimate for the shortest distance from the Cu atom to the solvent in the direction of the His 87 imidazole ring in plastocyanin.

Predictions concerning the Cu ligand groups were less unanimous than those concerning the geometry of the Cu site. The possibility of Cu-N(His) or Cu-S coordination was inferred³⁷ from the high redox potentials of the $\text{Cu}^{2+}/\text{Cu}^{+}$ couple in aqueous solutions containing Cu complexes of imidazole or a thioether ligand³⁶. A firm prediction that two His residues are present among the Cu ligands in plastocyanin was made on the basis of identifiable differences between the proton NMR spectra of Cu(I)- and Cu(II)-plastocyanin⁴⁵. The first evidence that the Cu atom in plastocyanin is coordinated by the single Cys thiol group was chemical: it was found that Cu-plastocyanin can be reconstituted by adding CuSO_4 to apoprotein, but not by adding CuSO_4 to apoprotein treated with a thiol-specific reagent^{46,47}. Physical evidence for Cu-S(Cys) binding was obtained from the electronic spectra of Cu(II)-plastocyanin and Co(II)-substituted plastocyanin³⁹, and by X-ray photoelectron spectroscopy (ESCA)^{48,49}. (It has been noted that the changes in S $2p_{3/2}$ binding energy observed in the ESCA experiments were significantly larger than those found for other metal complexes with sulphur-containing ligands⁶⁴.) It was consistent with Cu-S(Cys) binding to identify the charge-transfer component of the intense absorption bands of 'Type I' Cu-proteins near 600 nm as a S→Cu transition^{39,40}. Similar charge-transfer bands with high absorbances at 500–600 nm were observed for a number of Cu(II)-thiolate complexes where Cu-S coordination was demonstrated by crystal structure analysis⁵⁰⁻⁵². In the absence of the information that the metal atom in plastocyanin forms two Cu-S and two Cu-N bonds, resonance Raman frequencies in the 250–470 cm^{-1} range were attributed to the vibrational modes of a Cu-S bond ($\sim 260 \text{ cm}^{-1}$) and three or four Cu-N and Cu-O bonds (350–470 cm^{-1})^{27,53}. The assignments differed slightly depending on whether the cooperation geometry was treated as tetrahedral²⁷ or trigonal-bipyramidal⁵³.

Thus the chemical natures of three of the Cu ligands in plastocyanin—His 37, His 87 and Cys 84—could reasonably be expected on the basis of experimental data derived from the protein and from model compounds. Proposals that the fourth donor atom is either the deprotonated nitrogen of a backbone peptide group in a helical segment of the molecule⁵⁴, or the phenolate oxygen of a Tyr side-chain⁵⁵, are shown to be incorrect

by our work. The only experimental data to suggest that Met is the fourth ligand came from model compounds. Coordination of Cu by S(Met) rather than S(Cys) was said to be favoured by the properties of a series of Cu(II) complexes with thioether ligands⁵⁶. The complexes had two of the essential properties of 'Type I' Cu-proteins: intense electronic absorption bands⁵⁶ (assigned to thioether S→Cu charge-transfer⁵⁷) and high redox potentials²⁴ (consistent with the greater affinity of thioether groups for Cu(I) than for Cu(II)⁵⁸). It is true that the conservation of Met 92 in plastocyanins from a wide variety of sources (and the conservation of an equivalent Met in azurins) pointed to a functional importance for this residue. This was realised by several workers²⁸⁻³⁰, but doubts were created by a report that the plastocyanin from dock (*Rumex obtusifolius*) contains no Met residues⁵⁹. A recent revision of the amino acid sequence of dock plastocyanin includes Met at the expected position⁶⁰. We note that tentative proposals based on sequence homology were also made for Cu binding at His 37 (refs 3,28), His 87 (refs 3,28,29) and Cys 84 (refs 3,28–30).

A more ambitious use of amino acid sequence data was represented by an attempt to predict the most probable values of all the torsion angles between the backbone peptide groups of plastocyanin, and hence the three-dimensional structure⁶¹. Even at the present stage of the structure analysis, a large proportion of the elements of secondary structure predicted by the calculation (Table 1 of ref. 61) are seen to be correct. The success rate in predicting the tertiary structure was low, as anticipated⁶¹. The application of an established curve-fitting procedure to the ultraviolet circular dichroism spectrum of plastocyanin⁵⁴ seriously underestimated the proportion of β structure in the molecule.

Finally, the structural results provide a reasonable explanation for the pH dependence of the redox potential of plastocyanin. The observed⁶² increase of about 60 mV per pH unit below pH 5.4 shows that a ligand group with a pK_a near 5 (probably imidazole) becomes dissociated from the Cu atom at low pH³⁷. The ligand involved in this reaction is probably His 87. The outer edge of the imidazole ring of His 87 is exposed to the solvent (Fig. 4) whereas His 37 is relatively buried in the protein. Dissociation of His 87 could leave the Cu with NS_2 coordination and presumably with a distorted trigonal geometry which further stabilises Cu(I) with respect to Cu(II). This hypothesis accounts for the high redox potential of plastocyanin at low pH, and is consistent with observations (A. G. Sykes, personal communication) that Cu(I)-plastocyanin becomes redox-inactive with respect to $\text{Fe}(\text{CN})_6^{3-}$ at pH below approximately 4.3.

A preliminary account of the structural results reported in here appeared in ref. 63. The participation of T. Bohdanowicz, D. J. Fensom, M. Smith and E. Woodcock in the crystallisation experiments is gratefully acknowledged. This work was supported by the Australian Research Grants Committee (grant 74/15398), and by a General Development Grant from the University of Sydney. P. M. Colman was a Queen Elizabeth II Fellow. We thank Drs A. G. Sykes and R. Ambler for communicating results prior to publication, Professors J. Drenth and R. Huber and their colleagues for providing computer programs, and Dr J. K. Beattie, Professor B. Bosnich, Dr P. E. Wright and Mrs C. E. Wright for helpful discussions.

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Localisation of a male-specific DNA fragment to a sub-region of the human Y chromosome

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A 'male-specific' DNA sequence has been localised to human satellite III DNA of the Y chromosome.

REPEATED DNA sequences that are specific to human male cells, and by implication, specific to the human Y chromosome, have been identified by two different methods^{1,2}. Kunkel *et al.* re-associated DNA from a male in the presence of a large excess of female-derived DNA and showed that the male DNA sequences, which remained single stranded, would undergo reassociation with DNA from a male, but not with DNA isolated from a female. Further experiments³ using DNA isolated from cells containing aberrant Y chromosomes, suggest that these 'male-specific' DNA sequences isolated by 'exclusion hybridisation' are restricted to the long arm of the Y chromosome. Using a different approach Cooke¹ digested male DNA with the restriction endonuclease *Hae*III and, following gel electrophoresis, identified two bands that represented DNA fragments of 2.24×10^6 and 1.6×10^6 molecular weight, respectively, and which were not present in *Hae*III digests of human female DNA. The proportions of the male specific DNA fragments were increased in cells with a 47, XYY chromosome constitution, further supporting the notion that these DNA sequences were related to the presence of the Y chromosome.

In a series of studies on the sequence homologies of the four human satellite DNAs, I, II, III, and IV⁴⁻⁷, we have identified DNA sequences that are only present in satellite III from human males and appear to be the same as the male-specific DNA identified by Cooke¹ in total human DNA. This report describes the association of these 'male-specific' sequences with human satellite III, the relationship between their sequence and the remainder of the satellite III DNA sequences and their localisation in the human Y chromosome.

Male-specific DNA in satellite III

When human satellite III DNA from female cells is digested to completion with *Hae*III restriction endonuclease and the resulting fragments separated according to size by agarose gel electrophoresis, a complex pattern of fragments is revealed (Fig. 1). The smallest clear band is about 200 nucleotide pairs in length, and the higher molecular weight bands are sizes which are simple

multiples of 200 nucleotide pairs. The largest band present in female satellite III DNA, which forms the upper limit of the multimeric series, is composed of DNA fragments 2,200 nucleotide pairs in length. There are no significant fragments of sizes between the 2,200-nucleotide pair fragment and the undigested DNA that remains close to the gel origin. Figure 1 shows the distribution of sizes of fragments obtained by digestion with *Hae*III of satellite III DNA from male cells. The sizes of fragments of 2,200 nucleotide pairs and less are essentially identical to those present in female satellite III DNA. The striking difference is the constant presence of a prominent 3,500 nucleotide pair (3.5 kilobases) band and occasionally the presence of a minor 2,600 nucleotide pair band, in the satellite III isolated from male cells. The larger of these bands can be routinely identified in *Hae*III digests of total DNA from male cells (Fig. 1) in agreement with the findings of Cooke¹, whereas we have not been able to consistently detect the smaller male-specific fragment in digests of total DNA from males. There is no indication that fragments of these sizes are present in total DNA from female cells. The 3.5-kilobase 'male' fragment is also present in male satellite II sequences: we have not detected it in either satellites I or IV (C.J.B. & A.R.M., unpublished data).

We have attempted to estimate the proportion of the male-specific fragment of size 3.5 kilobases present in male DNA by densitometry using photographic negatives of *Hae*III restriction digests of total DNA, with or without known amounts of added DNA of known fragment size, or by densitometry of *Hae*III digests of isolated satellite III DNA. Considering only male satellite III, the 3.5 kilobase male fragment comprises about 10% of this satellite, and since this satellite represents 0.75% of the total genome⁸, this suggests that the 3.5 kilobase male-specific fragment comprises 0.075% of the genome. This latter figure is low in comparison to the estimate derived from total male DNA, where the average of several different estimates indicated that the 3.5 kilobase male-specific fragment comprised $0.1 \pm 0.008\%$ of the genome. However, it should be remembered that DNA satellite II contains a proportion of the 3.5 kilobase male band. While both of these methods cannot be considered accurate due to several possible sources of error, they do suggest that the larger of the male-specific bands comprises about 0.1% of the total cellular

DNA—equivalent to approximately 15% of the DNA contained in the Y chromosome. Using this figure, the 3.5 kilobase male-specific fragment would be composed of about 1,000 copies of a sequence 3,500 nucleotide pairs in length.

Location on the Y chromosome

The human Y chromosome contains a large region at the distal end of the long arm which fluoresces intensely after staining with quinacrine and stains positively with the C-banding technique. The Y chromosome has also been shown to hybridise *in situ* all four human satellite DNA sequences, which are also located in the C-band regions of several other human chromosomes⁹. It is, therefore, of interest to know (1) whether this male-specific DNA fragment contains a sequence that can be located only on the Y chromosome, (2) the position it occupies on the Y chromosome and (3) whether it contains the same basic repeated sequence as the remainder of human satellite III. The apparent distinctiveness of the fragment size could merely represent a particular spacing of the *Hae*III recognition site in an otherwise identical DNA sequence.

In principle hybridisation *in situ* is ideally suited to answer the first of the above questions, but the limits of resolution of this technique will not permit the delineation of where on the Y chromosome the male-specific DNA fragment may be located. Results obtained from *in situ* hybridisation of the various DNA fragments obtained by *Hae*III digestion of human satellite III are complex, and will be reported in detail elsewhere (our work, in preparation). Briefly, sequences, which are homologous to those contained in the 3.5-kilobase male-specific fragment, are found to be preferentially localised in the Y chromosome. Figure 2 shows the distribution of silver grains in autoradiographs of human metaphase chromosome spreads which had been hybridised *in situ* to ³H-cRNA made on a template of purified 3.5-kilobase male-specific fragment. In both short and long exposures the major site of hybridisation of the 3.5-kilobase fragment is to the long arm of the Y chromosome. This confirms its presence in the Y chromosome. In autoradiographs exposed for longer intervals, low but significant levels of hybridisation to specific autosomes (chromosomes 9 and 15) are also detected. Thus a sequence that is totally or partially homologous to that of the 3.5-kilobase fragment is present on some autosomes; this is consistent with the detection of this sequence in female DNA.

We have approached the question of where the male-specific DNA fragment is located on the Y chromosome by looking for its

Fig. 2 *In situ* hybridisation of ³H-RNA complementary to the 3.5-kilobase fragment of a *Hae*III digest of male satellite III. Satellite III DNA, 100 µg, from a male placenta was digested with *Hae*III and fractionated on a 1.5% Agarose gel (see legend to Fig. 1). The male-specific fragment was excised and recovered from the gel by diffusion into 0.01M Tris-HCl pH 7.9 (ref. 19). The DNA was concentrated by centrifugation and redissolved in 20 µl of the same buffer. The total DNA recovered (6 µg) was used as a template for cRNA synthesis by *E. coli* RNA polymerase⁹. *In situ* hybridisation to fixed human metaphase chromosomes was carried out as described previously⁹ and the autoradiographs were exposed for 90 (solid line) or 160 d (broken line). The results were analysed as the average number of grains per chromosome segment⁹ and plotted in the form of a histogram, with the centromere of each chromosome indicated on the abscissa. After 90 day exposure grains were almost exclusively found on the Y chromosome, but the longer exposure shows some additional sites, in particular chromosomes 9 and 15.

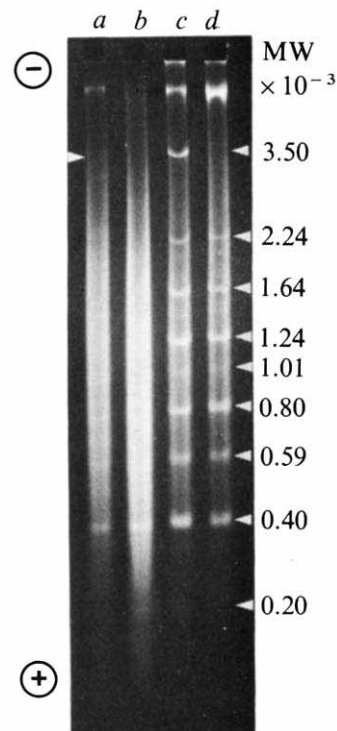
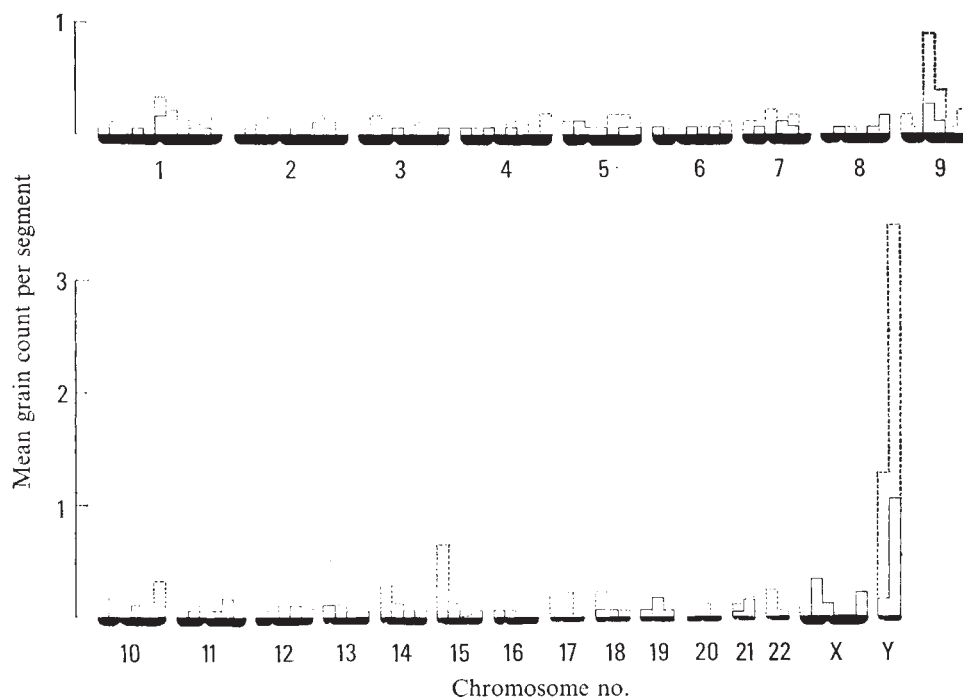


Fig. 1 *Hae*III restriction endonuclease digestion of purified satellite III and total DNAs from normal male and female cells. *a*, Total male (5 µg); *b*, total female (5 µg); *c*, male satellite III (2 µg); *d*, female satellite III (2 µg). The figures on the right refer to the molecular weights of the DNA fragments. DNA was purified from human placenta by a modification of Marmur's procedure¹³ and satellite III was isolated from it by caesium salt density gradient centrifugation as described elsewhere⁹. DNA was digested with *Hae*III restriction endonuclease (Biolabs) at a DNA to enzyme ratio of 1 µg-DNA to 1 unit of enzyme (1 unit of enzyme will degrade 1 µg of λ -phage DNA in 60 min at 37 °C in a total assay mixture of 50 µl, in 6 mM Tris-HCl (pH 7.4), 6 mM NaCl, 6 mM MgCl₂ and 6 mM 2-mercaptoethanol for 6 h at 37 °C. After digestion sucrose was added to 5% and the samples applied to the wells of a 1.5% Agarose slab gel. The 20 × 20 × 0.3 cm slab gel was made up and run in 40 mM Tris-HCl, 20 mM sodium acetate and 1 mM NaEDTA with the pH adjusted to 8.2 with acetic acid and run at 10 mA, 60 V for 15 h. The gel was stained with ethidium bromide (0.5 µgml⁻¹) and photographed using exciting light at 254 nm. Fragments were sized by comparison with the mobilities of *Eco*RI restriction digests of λ -phage DNA¹⁴ and *Eco*RII restriction partial digests of mouse satellite DNA¹⁵.

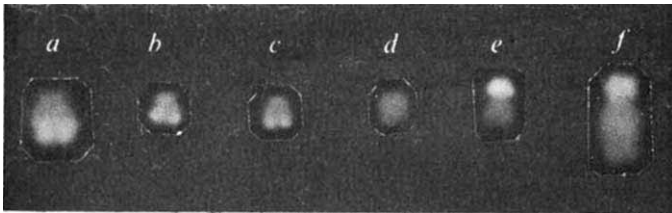


Fig. 3 Examples of quinacrine fluorescent banding of aberrant human Y chromosomes. *a*, Normal 46, XY male; *b*, skin culture 228; *c*, skin culture 6925. (*b*) and (*c*) were derived from 46, XY males carrying Y chromosome variants with small quinacrine-bright fluorescent segments on the distal end of Yq; *d*, skin culture DEV from a 46, XY, del(Y)(q11) individual, a terminal deletion of the entire quinacrine-bright fluorescent segment of Yq; *e*, lymphocyte culture from a 46,XX,der(22)t(22;Y)(p13;q12) phenotypically normal female showing the putative distal quinacrine-bright segment of Yq translocated to the short arm of chromosome 22; *f*, lymphocyte culture from a 46,XX,der(15)t(15;Y)(p12;q12) phenotypically normal female showing the putative distal quinacrine-bright fluorescent segment of Yq translocated to the short arm of chromosome 15. The skin culture 6925 was provided by D. Harnden.

presence or absence in DNA isolated from cells carrying various deletions of the Y chromosome. Examples of these are shown in Fig. 3. On the left is a normal Y chromosome showing the large distal bright fluorescent band. Moving to the right there are three examples of Y chromosome variants with decreasing amounts of the distal quinacrine bright material. The Y chromosome of DEV cells (Fig. 3*d*) completely lacks any quinacrine bright or C-band material and thus carries a deletion for band Yq12. Figure 3*e* and *f* shows two examples of translocations of the distal portion of the Y chromosome on to an autosome, in the one case to a chromosome 22 and in the other case to a chromosome 15. In the Y/autosome translocations DNA was extracted from cells of phenotypically normal females who carry the translocations. This permitted the examination of the quinacrine bright region of the Y chromosome in isolation from the non-fluorescent portion. The converse is true for the 'mini-Y' completely lacking fluorescence in male cells. The mini-Y carrying small amounts of fluorescence provides an intermediate situation.

Attempts to identify the larger male-specific fragment in *Hae*III digests of total DNA from the cells with abnormal Y chromosomes by ethidium bromide staining of Agarose gels showed that it could be detected only in DNA from normal males and in DNA from two mini-Y cells that contained some fluorescence. Cell 228 is an example of one of these mini-Y cells and is shown in Fig. 4*c*. The 3.5-kilobase male-specific DNA fragment was also identified in nearly normal proportions in female cells carrying the Y/15 translocation, (Fig. 4*e*). The 6925, DEV and Y/22 translocation showed no detectable 3.5-kilobase male-specific band. It is doubtful whether this technique would detect the presence of the 3.5-kilobase band if it is significantly reduced below its normally small percentage. Ethidium bromide stained gels do show that loss of large amounts of the quinacrine bright region of the Y chromosome result in a reduced amount of the male band, but its ready identification in one of the mini-Y cells (cell 228) shows that it is not lost in proportion to the amount of the quinacrine bright material, an observation which is consistent with it being located at least partly in the nonfluorescent region of the Y chromosome and in the region of the fluorescent band which is most proximal to the centromere.

To overcome the lack of sensitivity noted above, we have used both single¹⁹ and two-dimensional gel electrophoresis/hybridisation transfer methods^{10,17} to detect sequences homologous to the 3.5-kilobase fragment of male satellite III. The two-dimensional method also allows the investigation of homology between the DNA sequences contained in all the different sized fragments produced by *Hae*III digestion of satellite III.

When ³²P nick-translated *Hae*III-digested male satellite III is hybridised to a nitrocellulose filter containing unlabelled *Hae*III-digested male satellite III, three features are apparent (Fig. 5*a*). First, the DNA sequences of fragments below 2.2 kilobases are sufficiently homologous to form a distinctive matrix of spots

corresponding to the points where the ³²P nick-translated bands cross the unlabelled bands and undergo hybridisation. Additional smaller rows of spots can be detected and these correspond to the cross-hybridisation of DNA fragments present in smaller quantities in the *Hae*III-digested satellite III DNA. Second, there is no significant hybridisation between the ³²P nick-translated male band of 3.5 kilobases and any of the fragments of 2.2 kilobases or less in the unlabelled dimension. Nor is there hybridisation of the ³²P nick-translated DNA of 2.2 kilobases or below by the unlabelled male band. The DNA sequence present in this male-specific fragment is therefore not homologous to the majority of satellite III which contains the *Hae*III recognition site. Some hybridisation occurs along the lines of both the ³²P-labelled and unlabelled male bands at molecular sizes in excess of 3.5 kilobases and a faint tail is seen along the line of the ³²P-labelled band below 3.5 kilobases. The former probably represents incompletely digested DNA, present in sufficiently small amounts to go undetected by ethidium bromide fluorescence staining, or multimeric forms of the 3.5-kilobase male band. There is a suggestion of distinct localisation of hybridisation to DNA bands above the 3.5-kilobase fragment and although we have been unable to size them accurately in this procedure, they are consistent with being multimeric forms of the 3.5-kilobase fragment. A similar phenomenon was observed by Cooke¹ in autoradiograms of gels to which cRNA to the larger male band had been hybridised.

The third feature shown by these two-dimensional hybridised gels is that the material which remains as high molecular weight

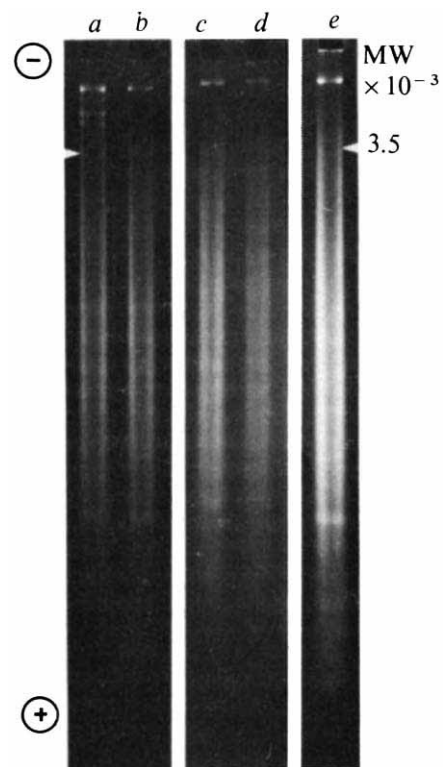


Fig. 4 Ethidium bromide stained 1.5% Agarose gels of *Hae*III restricted total DNAs from cells carrying different aberrant Y chromosomes. From left to right: *a*, normal male DNA (4 µg) mixed with 0.5 µg λ-phage DNA digested with *Eco*RI restriction enzyme. Note the co-migration of the smallest λ-phage restriction fragment with the male-specific fragment of male DNA; *b*, normal male DNA (4 µg); *c*, cell 228 DNA (4 µg); *d*, Y/22 translocation lymphocyte DNA; *e*, Y/15 translocation lymphocyte DNA. The 3.5-kilobase fragment was detected in (*a*), (*b*), (*c*) and (*e*), but not in (*d*). Skin fibroblast cultures were grown in Eagle's minimal essential medium, supplemented with 10% foetal calf serum and containing penicillin and streptomycin. Cells were collected by scraping off the monolayer with a rubber policeman. Blood lymphocytes were separated from erythrocytes¹⁶ and cultured at 10⁶ cells ml⁻¹ in Ham's F10 medium, supplemented with 15% pooled human serum, and stimulated with 1% phytohaemagglutinin (HA15 Wellcome). The cells were collected after 96 h. Preparation of DNA, restriction endonuclease digestion and gel electrophoresis were as described in legend to Fig. 1.

DNA after digestion with *Hae*III, and therefore only partially enters the gel, is not homologous to the fragments of 2.2 kilobases or less. It does, however, contain sequences that are homologous

that they did not contain any human DNA in the unlabelled dimension—do not produce detectable autoradiograms in the time required to produce autoradiograms of unlabelled DNA

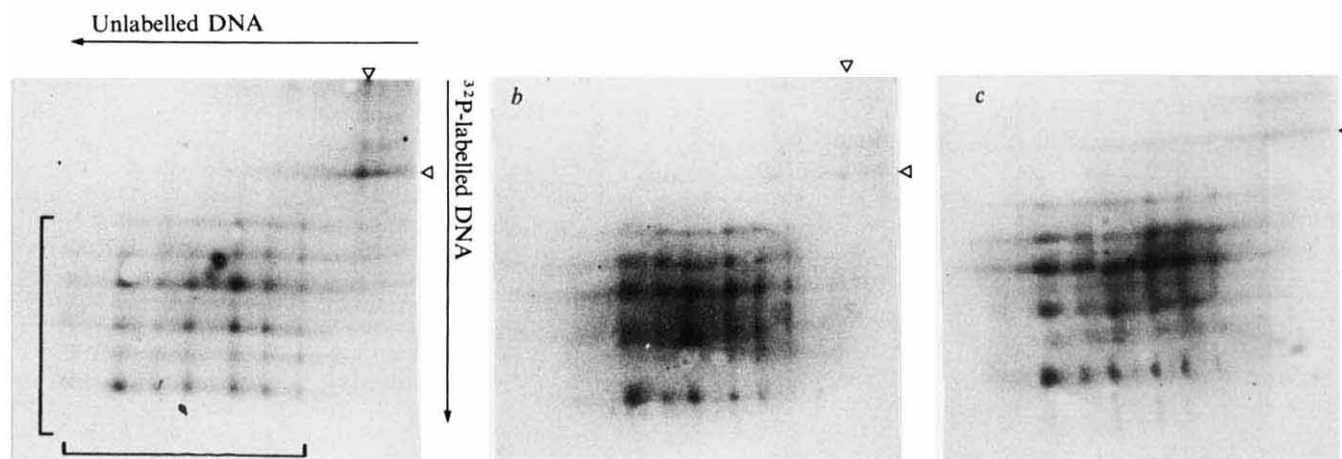


Fig. 5 Autoradiograms of two-dimensional gel electrophoresis/hybridisation transfers of *Hae*III-digested male satellite III (a) and male (b) and female (c) total DNAs. The method of Hutchison^{10,11} was followed without significant modification. Briefly, *Hae*III-digested, unlabelled DNA was separated by gel electrophoresis as described in Fig. 1, except that the sample was loaded in a single well that occupied the entire width of the gel. After electrophoresis the separated fragments were denatured in the gel, neutralised and transferred to a nitrocellulose filter square 20 × 20 cm. *Hae*III-digested male satellite III was labelled with α -³²P-thymidine triphosphate by nick translation¹⁸ to a specific activity of about 2×10^7 d.p.m. μg^{-1} . The labelled fragments were separated by electrophoresis as described above, and the fragments transferred to the nitrocellulose filters, containing the unlabelled DNA samples, under hybridising conditions after the fragments had been denatured and neutralised in the gel. After washing the filters in 3 mM Tris base at room temperature and 0.12 M Na phosphate buffer at 70 °C, the filters were autoradiographed using Kodak X-omat H X-ray film. The hybridisation transfer of ³²P nick-translated DNA was carried out after rotation of the filter square through 90° from the position at which transfer of the unlabelled fragments took place. Thus at some point on the filter each band of ³²P nick-translated male satellite III DNA fragments will cross each band of unlabelled DNA fragments bound to the filter. The positions of areas of retained radioactivity indicate hybridisation resulting from the homology of the sequences where the labelled and unlabelled fragments crossed. The direction of electrophoresis of each DNA sample is indicated by the closed arrows. The open arrows indicate the positions of 3.5-kilobase fragment. The brackets indicate the matrix formed by the cross-hybridisation of fragments of 2.2 kilobases and below.

to the 3.5-kilobase male fragment. Although DNA of this molecular weight does not transfer efficiently out of Agarose gels, enough was eluted to give a strong spot representing hybridisation at the point where the two gel origins meet. If sequences homologous to those digested by *Hae*III were present in the high molecular weight DNA, we would have expected to have seen equally strong spots where each individual band crossed the undigested material.

A similar autoradiogram is obtained if unlabelled, *Hae*III digested, total male DNA is used in the unlabelled dimension in place of purified satellite III, except that the 'background' hybridisation is greater (Fig. 5b). In contrast, unlabelled *Hae*III-digested total female DNA (Fig. 5c) shows distinctive differences in the region of the male band, although the matrix produced by cross-hybridisation of fragments of 2.2 kilobases or less is unaltered. There is apparent hybridisation along the length of the 3.5-kilobase fragment present in the ³²P-labelled nick-translated male satellite III, but there is no hybridisation to any discrete fragment in the dimension of the unlabelled female DNA at the size expected for the 3.5-kilobase male-specific DNA. This shows that in female DNA there is no discrete set of fragments, 3.5 kilobases in length, that contain sequences homologous to those present in the 3.5-kilobase fragment of *Hae*III-digested male satellite III. Hybridisation along the line of the ³²P-labelled nick-translated male fragment indicates either that female DNA does contain homologous sequences (but that these occur in fragments covering a wide range of sizes after *Hae*III digestion) or that the radioactive single strands of DNA are binding nonspecifically to the nitrocellulose filter during the hybridisation transfer. We cannot eliminate the latter possibility entirely, but we feel that it does not contribute significantly to the binding of radioactivity for the following reasons. First, blank filters—filters processed and 'hybridised' in exactly the same way as sample filters except

samples. Second, the amount of radioactivity bound along the length of the ³²P-labelled band always decreases in the direction of decrease in fragment size in the cold dimension; that is, binding is related in some way to the amount of unlabelled human DNA on the filter rather than the amount of radioactive DNA. Third, pre-incubation of the filter, in a solution of Ficoll, polyvinylpyrrolidone and bovine serum albumin¹¹, which reduces the non-specific binding of DNA to nitrocellulose filters during the hybridisation step, does not reduce the level of binding during the hybridisation transfer. Finally the band of bound radioactivity is resistant to washing in 0.12 M phosphate buffer at 60 °C, partially resistant at 76 °C, whereas it is removed by washing at 100 °C. This is characteristic of the mixture of partially mis-matched and well matched duplex molecules contained in re-associated human satellite III DNA sequences (A.R.M. & C.J.B., unpublished). If this assumption is correct then the essential difference between male and female DNA is that, while they both contain sequences homologous to some or all of those present in the 3.5-kilobase fragment of male satellite III, only in the male DNA are they repeated a thousand or so times in DNA which contains the *Hae*III recognition site once every 3,500 nucleotide pairs. The question of location, therefore, must be limited to a consideration of the DNA sequence that contains this spacing of the *Hae*III recognition site.

The male fragment in aberrant Y chromosomes

Fluorescent ethidium bromide stained gels of *Hae*III-digested total DNAs suggest that the male-specific 3.5-kilobase fragment is reduced in amount, or absent, from three of the four mini-Y chromosome individuals and the individuals carrying a putative Y22 translocation. We have used the single-dimension hybridisation technique¹⁹ to find whether the DNAs from individuals carrying these aberrant Y chromosomes contain a DNA

fragment 3.5 kilobases in length, which is homologous to the male fragment of human satellite III. For this experiment unlabelled total DNA from each of these individuals was digested with *Hae*III and the resulting fragments separated in an Agarose gel. The fragments were transferred to nitrocellulose and hybridised to ³²P nick-translated, male satellite III. Figure 6 shows the autoradiogram of the resultant hybridised filters. Hybridisation



Fig. 6 Detection of the 3.5-kilobase male-specific fragment in DNAs isolated from cells carrying aberrant Y chromosomes. *a*, Normal male DNA; *b*, normal female DNA; *c*, DEV cell DNA; *d*, A127 cell DNA; *e*, 6925 cell DNA; *f*, Y/15 translocation female cell DNA; *g*, Y/22 translocation female cell DNA. 3 μ g of each DNA was digested with 1 unit of *Hae*III restriction enzyme at 37 °C for 16 h. The resulting fragments were separated by electrophoresis in a 1.5% Agarose slab gel and transferred to a nitrocellulose filter after alkaline denaturation²⁰. The filter was hybridised to ³²P nick-translated male satellite III (0.25 μ g; 2×10^7 d.p.m. μ g⁻¹) in $3 \times$ SSC containing 0.1% SDS at 65 °C for 20 h. After washing at 65 °C in several changes of $2 \times$ SSC containing 0.1% SDS the filter was autoradiographed using Kodak X-omat H X-ray film. The gel tracks on the extreme left and right are fragments of λ DNA resulting from digestion with *Hind*III and *Eco*RI restriction enzymes. Their locations were detected by the inclusion of ³²P nick-translated λ DNA in the hybridisation solution and were used as size markers to identify the position of the 3.5-kilobase fragment in the human DNAs. The arrows indicate the position of the 3.5-kilobase male-specific fragment. Cell A127 carries a Y chromosome variant with a small distal quinacrine-bright fluorescent band, similar to that present in cell 6925 (see Fig. 3).

to a 3.5-kilobase fragment is clear in the DNA from cells containing a normal Y chromosome (*a*), two cells carrying mini-Y chromosomes (*d* and *e*), the DEV cell carrying the deletion of the entire distal Yq12 band (*c*), and the female cell carrying a Y/15 translocation (track *f*). No hybridisation is seen to a 3.5-kilobase fragment in DNA from normal female cells (track *b*) or from female cells carrying the Y/22 translocation (track *g*), despite normal levels of hybridisation to the other DNA fragments with sizes characteristic of *Hae*III-digested satellite III.

On the basis of these results one can tentatively assign the 3.5-kilobase male-specific fragment a location on the Y chromosome. The Y chromosome of the DEV cells carries a deletion of the entire distal Yq12 band. Such a deletion results in the reduction in the amount of the 3.5-kilobase fragment such that it is not visible in ethidium bromide stained gels of *Hae*III-digested total DEV DNA. The detection, by hybridisation, of its presence in DEV DNA shows that some of the 3.5-kilobase repeated sequence must be located in the nonfluorescent part of the Y chromosome. In cell line 228, in which only a small amount of band Yq12 is present, a nearly normal amount of the 3.5-kilobase fragment is observed. This suggests that the bulk of DNA containing the 3.5-kilobase spacing of the *Hae*III recognition site is located at the proximal end of band Yq12. This location is also consistent with the results from the Y/autosome translocations, since its presence in the Y/15 translocation could reflect the translocation of the entire Yq12 band, whereas its absence in the Y/22 translocation could reflect the translocation of only the terminal portion of band Yq12, from which the 3.5-kilobase repeating sequence is missing. Alternatively, it is possible that the Y/22 translocation does not represent the translocation of Y chromosome material, but instead represents a unique addition to the autosomal complement, for example, a giant-satellited chromosome arm. The latter is unlikely, since its staining properties in several cytogenetic techniques show that the extra portion on chromosome 22 behaves precisely as though it is part of band Yq12 (ref. 12 and A. C. Chandley, personal communication) and not in a way characteristic of the satellite regions of chromosomes. A non-random distribution of the 3.5-kilobase fragment in the Y chromosome is also consistent with its disproportionately low increase in a male carrying a Y chromosome with extensive reduplication of the Yq12 region (H. Cooke personal communication).

In summary, the results presented here show that the 3.5-kilobase DNA fragment, identified after digestion with *Hae*III restriction endonuclease, co-purifies with human satellite III. Part (or all) of the repeated DNA sequence between the *Hae*III recognition sites is sufficiently homologous to DNA sequences present on autosomes to undergo hybridisation *in situ* or in the conditions used in the hybridisation transfer and subsequent washing. Despite homology of sequence between DNA of the 3.5-kilobase male-specific fragment and that in the autosomes, the 3.5-kilobase spacing of the *Hae*III recognition site seems to be unique to the Y chromosome. The experiments on aberrant Y chromosomes suggest that the 3.5-kilobase DNA fragment is located in the nonfluorescent portion, and in the most proximal part of the fluorescent portion (band Yq12) of the Y chromosome.

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Brain histamine receptors as targets for antidepressant drugs

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A large number of structurally diverse drugs with clinical antidepressant properties share the ability to act as potent inhibitors of histamine-sensitive adenylate cyclase in cell-free preparations from mammalian brain. This common biochemical action may represent part of the molecular basis for the antidepressant properties of these compounds.

THE tricyclic antidepressant drugs, a class which includes imipramine, amitriptyline, desmethylinipramine and doxepin, have achieved widespread clinical use in the treatment of depression. However, the molecular basis for their antidepressant properties is not known. These drugs do possess activity on diverse pharmacological systems¹; for example, many are potent antagonists of muscarinic acetylcholine receptors²⁻⁴, histamine H₁-receptors^{2,3}, serotonin receptors^{2,3} and dopamine receptors⁵. However, these

actions are not thought to be responsible for their antidepressant properties, for antagonists of these receptors other than the tricyclic antidepressant drugs do not possess clinical antidepressant activity.

Two pharmacological properties of the tricyclic antidepressant drugs that have been extensively investigated are their ability to potentiate the synaptic actions of noradrenaline (NA) and those of serotonin (5-HT) in the nervous system by blocking the biogenic amine reuptake systems present in presynaptic nerve endings⁶⁻¹⁴. Tricyclic antidepressant drugs which are secondary and tertiary amines, respectively, are relatively selective inhibitors of NA^{8,11,14} and 5-HT^{7,11,14} reuptake, and these actions may be related to their therapeutic antidepressant effects. Carlsson *et al.*^{7,8} have suggested that the blockade of 5-HT reuptake may be responsible for the ability of these drugs to elevate the mood of depressed patients, and that the blockade of NA reuptake may account for their ability to alleviate the motor retardation observed in some depressed patients.

The results of studies on reuptake mechanisms provide important evidence in support of the hypothesis that abnormalities in the noradrenergic and serotonergic systems in the brain may be involved in the aetiology of affective disorders¹⁵⁻¹⁹. However, the pharmacological properties of two newer 'atypical' antidepressant drugs have raised certain questions about the role of NA and 5-HT in affective disorders. Iprindole, structurally an *N*-substituted cycloalkylindole, has little or no effect on the NA²⁰⁻²⁴ and 5-HT reuptake^{22,23} processes in neurones, while mianserin also has little effect on NA and 5-HT reuptake^{25,26}, and is, in fact, a 5-HT receptor antagonist²⁷. Yet, both iprindole²⁸⁻³¹ and mianserin^{32,33} have been shown to be highly effective as antidepressant drugs in several clinical studies. The molecular basis for their clinical activity is not known. We therefore investigated the possibility that the clinical antidepressant properties of the tricyclic antidepressant and the 'atypical' antidepressant drugs might be a consequence, at least in part, of their actions on other biogenic amine systems in the brain. The present study concerns the interactions of various classes of drugs which have antidepressant properties with histaminergic systems in the mammalian brain.

Effects of antidepressant drugs on a histamine-sensitive brain adenylate cyclase

There is increasing evidence that histamine may be a neurotransmitter in the mammalian central nervous system³⁴. An ascending histaminergic pathway in rat brain has been described³⁵ which passes through the medial forebrain bundle and has a diffuse projection over the entire telencephalon. In non-neuronal tissues, histamine can exert its physiological effects by interacting with either of two types of receptors, designated H₁ and H₂^{36,37}. In the central nervous system, histamine, through activation of H₂-receptors, depresses the firing rate of certain neurones in the cerebral cortex³⁸. We have recently described a histamine-sensitive adenylate cyclase present in homogenates of guinea pig³⁹ and human (unpublished results) hippocampus and neocortex which has pharmacological properties similar to those of the H₂-receptor. The activation of this adenylate cyclase by histamine was blocked by the H₂-receptor antagonist metiamide, but not by α -adrenergic, β -adrenergic, or muscarinic cholinergic antagonists³⁹. The highest specific activities of histamine-sensitive

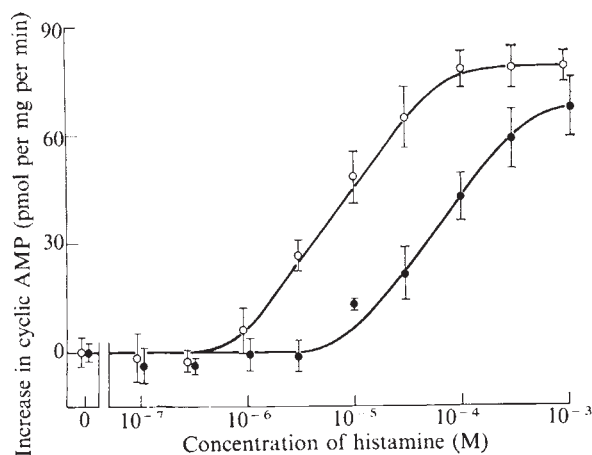


Fig. 1 Effect of various concentrations of histamine, in the absence (○) or presence (●) of 0.6 μ M chlorimipramine, on adenylate cyclase activity in homogenates of guinea pig hippocampus. Adult female Hartley guinea pigs (100–300 g) were killed by decapitation. The hippocampi of the brain were dissected, weighed and manually homogenised in 100 volumes of ice-cold 2 mM Tris-maleate (pH 7.8)—2 mM EGTA, using a glass homogeniser with a Teflon pestle. This preparation was used, within 30 min of homogenisation, as the enzyme source. The adenylate cyclase reaction mixture contained the following substances (final concentrations in mM): Tris-maleate (pH 7.8), 100; EGTA, 0.6; 3-isobutyl-1-methylxanthine, 1; MgCl₂, 2; ATP, 1; GTP, 0.1; plus histamine and test substance as indicated, in a final volume of 500 μ l. Following the addition of 50 μ l of the homogenate to the reaction mixture minus ATP, each assay tube was vortexed and incubated in an ice-water bath for 10 min. The adenylate cyclase reaction was then initiated by the addition of ATP, carried out for 6 min in a shaking water bath at 30°C, and terminated by placing the assay tubes in a hot water bath (93–98°C) for 3 min. 50 μ l aliquots of each sample were assayed in duplicate for cyclic AMP by the method of Brown *et al.*⁴². Protein was determined by the method of Lowry *et al.*⁴³. In the absence of added histamine or chlorimipramine, 83.8 $\pm$ 3.8 pmol cyclic AMP per mg protein per min were formed; in the absence of added histamine and presence of 0.6 μ M chlorimipramine, 85.6 $\pm$ 2.3 pmol cyclic AMP per mg protein per min were formed. The increase in cyclic AMP above basal level is plotted as a function of the histamine concentration. Values represent the mean $\pm$ s.e.m. of 6 replicate samples in the absence, and 3 replicate samples in the presence, of histamine.

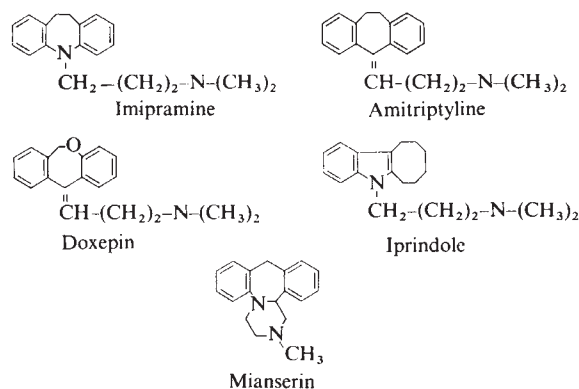


Fig. 2 Chemical structures of some antidepressant drugs.

adenylate cyclase were observed in subcellular fractions of brain rich in synaptic membranes⁴⁰. These results suggest that the H₂-receptor in the brain is coupled to an adenylylase, and that the activation of this enzyme by histamine may be an early step in the sequence of biochemical events through which histamine exerts certain of its physiological effects in the central nervous system. We now report that the classical tricyclic antidepressant drugs, as well as the 'atypical' antidepressant drugs iprindole and mianserin, and even certain phenothiazine and thioxanthene derivatives that possess antidepressant properties, are all potent antagonists of the activation of brain adenylylase by histamine. When these studies were near completion, Green and Maayani independently demonstrated inhibition of histamine-sensitive adenylylase by some antidepressant drugs⁴¹.

The effects of various concentrations of histamine on adenylylase activity measured in homogenates of guinea pig hippocampus are shown in Fig. 1. In many experiments of this type, the concentration of histamine required for half-maximal activation (K_a) of adenylylase was 7–9 μ M. Maximal stimulation was observed at 100–300 μ M histamine, and resulted in an approximately twofold increase in enzyme activity. Figure 1 also demonstrates that the antidepressant drug chlorimipramine acted as a competitive inhibitor of the activation of adenylylase by histamine. In the presence of 0.6 μ M chlorimipramine, the concentration of histamine required for half-maximal activation of the enzyme increased from 7 μ M to 84 μ M. From these data, the inhibition constant (K_i) of the enzyme for chlorimipramine was calculated⁴⁴ to be 0.055 μ M. The inhibition of histamine-sensitive adenylylase activity by many different antidepressant drugs was studied in further experiments of this type, in which the concentration of histamine was varied in the absence and presence of a fixed concentration of antidepressant drug. Every antidepressant drug studied, including representatives of each of five major chemical classes (Fig. 2), was a potent competitive inhibitor of the activation of adenylylase by histamine (Table 1a). The chemical classes of antidepressant drugs examined included dibenzazepines, dibenzocycloheptadienes, a dibenzoxepine, an *N*-substituted cycloalkylindole, and a tetracyclic piperazine. Chlorimipramine and amitriptyline were the most potent inhibitors of histamine-sensitive adenylylase found among these drugs. The tricyclic antidepressant drugs which are tertiary amines (chlorimipramine, imipramine, amitriptyline) were more potent inhibitors than those which are secondary amines (desmethylinipramine, nortriptyline, protriptyline). Of particular interest is the potent inhibition by the 'atypical' antidepressant drugs iprindole and mianserin. In fact, mianserin was one of the most potent inhibitors, while iprindole, although somewhat less potent, still had an inhibition constant for the enzyme nearly equal to that of imipramine.

The inhibition of histamine-sensitive adenylylase activity by antidepressant drugs was also studied in experiments in which the concentration of the drug was varied while the concentration of histamine was held constant at 100 μ M. Figure 3 shows the

effects of various concentrations of chlorimipramine, imipramine, and desmethylinipramine on enzyme activity measured in homogenates of guinea pig hippocampus. Low concentrations of each of these drugs blocked the activation of adenylylase by histamine. In contrast, basal adenylylase activity was not altered except at relatively high concentrations ($> 10^{-5}$ M). Experiments of this type were carried out for many different antidepressant drugs. For each drug, the concentration required for half-maximal inhibition of enzyme activity was determined, and the inhibition constant was calculated assuming competitive inhibition by the drug at the histamine receptor site. The results are summarised in Table 1b, and the inhibition constants obtained for each drug, as determined by the two experimental designs, were in close agreement (compare Table 1a with 1b). Also, using homogenates of guinea pig neocortex, the con-

Table 1 Calculated inhibition constants (K_i) of antidepressant drugs for histamine-sensitive adenylylase activity in homogenates of guinea pig hippocampus

(a) Drug	K'_a/K_a	K_i (μ M)
Dibenzazepine		
Chlorimipramine (0.6 μ M)	12.0	0.055
Imipramine (2 μ M)	13.9	0.16
Desmethylinipramine (4 μ M)	12.5	0.35
Dibenzocycloheptadiene		
Amitriptyline (1 μ M)	20.0	0.053
Nortriptyline (6 μ M)	14.4	0.45
Protriptyline (7 μ M)	15.6	0.48
Dibenzoxepine		
Doxepin (1.5 μ M)	10.0	0.17
<i>N</i> -substituted cycloalkylindole		
Iprindole (1.5 μ M)	7.5	0.23
Tetracyclic piperazine		
Mianserin (0.8 μ M)	13.3	0.065
(b) Drug	IC ₅₀ (μ M)	K_i (μ M)
Dibenzazepine		
Chlorimipramine	0.72	0.053
Imipramine	1.9	0.14
Desmethylinipramine	3.8	0.28
Dibenzocycloheptadiene		
Amitriptyline	0.66	0.049
Nortriptyline	7.0	0.52
Protriptyline	6.5	0.48
Dibenzoxepine		
Doxepin	1.6	0.12
<i>N</i> -substituted cycloalkylindole		
Iprindole	2.7	0.20
Tetracyclic piperazine		
Mianserin	0.88	0.065

(a) Data from experiments (of the type shown in Fig. 1) in which the concentration of histamine was varied and the concentration of antidepressant drug held constant. The inhibition constant (K_i) was calculated⁴⁴ according to the equation:

$$K_i = I / [(K'_a/K_a) - 1]$$

where K_a and K'_a are the concentrations of histamine required to give half-maximal activation of adenylylase in the absence and presence of antagonist, respectively, and I is the concentration of antagonist. The concentration of antidepressant drug used in each experiment is indicated in parentheses. At the concentrations used in these experiments, the antidepressant drugs had little or no effect on adenylylase activity measured in the absence of added histamine. (b) Data from experiments (of the type shown in Fig. 3) in which the concentration of histamine was held constant at 100 μ M and the concentration of antidepressant drug varied. The inhibition constant (K_i) was calculated⁴⁴, assuming competitive inhibition, according to the equation:

$$K_i = IC_{50} / (1 + S/K_a)$$

where IC_{50} is the concentration of antagonist required to give 50% inhibition of the histamine-stimulated increase in adenylylase activity, S is the concentration of histamine (100 μ M), and K_a is the concentration of histamine required to give half-maximal activation of adenylylase (8 μ M). Basal adenylylase activity was not altered over the range of concentrations of drugs used to determine the IC_{50} values.

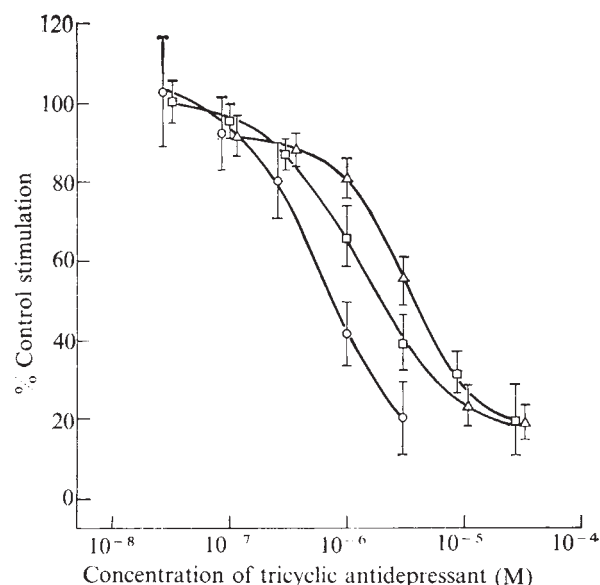


Fig. 3 Effect of various concentrations of chlorimipramine (○), imipramine (□), and desmethylinipramine (Δ) on adenylate cyclase activity measured in the presence of 100 μ M histamine, in homogenates of guinea pig hippocampus. In each experiment, the control stimulation (that is, the increase in adenylate cyclase activity observed in the presence of 100 μ M histamine) is defined as 100%, and is based on the measurement of 6 replicate samples in the absence, and 6 replicate samples in the presence of 100 μ M histamine. The increase in enzyme activity observed in the presence of 100 μ M histamine, at each concentration of antidepressant drug, is expressed as a % of the control stimulation. Each plotted value represents the mean $\pm$ s.e.m. of 3 replicate samples.

centration of histamine required for half-maximal activation of the enzyme, and the inhibition constant obtained for each antidepressant drug tested (Table 2), were similar to those values obtained in experiments in which the hippocampus was used as the enzyme source. These results indicate that the histamine-sensitive adenylate cyclases derived from these two brain regions possess similar pharmacological properties.

Application in a clinical context

The inhibition constants of the antidepressant drugs obtained *in vitro* are sufficiently low to suggest that the activation of adenylate cyclase by histamine may be markedly antagonised *in vivo* by pharmacologically effective doses of these compounds. Recent clinical studies have determined^{4,5} the range of plasma levels for certain tricyclic antidepressant drugs associated with a therapeutic response in depressed patients. Assuming that the steady-state ratios of brain/plasma concentrations of tricyclic antidepressant drugs are similar in the rat⁴⁶ and in man during chronic oral administration of these drugs, the brain concentrations of tricyclic antidepressant drugs associated with a therapeutic response in depressed patients can be calculated to be in the micromolar range. These calculated brain concentrations are significantly higher than the inhibition constants of these compounds for histamine-sensitive adenylate cyclase. Consequently, one might expect *in vivo* blockade of brain histamine receptors after administration of antidepressant drugs, with resultant physiological sequelae. Unfortunately, the physiological functions of histaminergic neurones and their postsynaptic receptors in brain are not known; no animal behavioural correlate reflecting the activity of brain histaminergic pathways has yet been described. However, in the present study we have observed, in a series of structurally diverse drugs that have therapeutic efficacy in the treatment of depression, that all act as potent inhibitors of brain histamine-sensitive adenylate cyclase. The members of this series do not share a known common site of action on any other single molecular process in neurones. Thus, the possibility is raised that certain of their therapeutic effects in the treatment of depression may be a consequence of their ability to block

histamine receptors in the brain. The results raise the further possibility that brain histamine may play a role in the biochemical mechanisms underlying depression.

There is generally little correlation between the potency of antidepressant drugs as inhibitors of brain histamine-sensitive adenylate cyclase and the average clinical dose used in the treatment of depression. However, differences in drug distribution, rates of metabolism⁴⁷, and therapeutic efficacy of metabolites, as well as the difficulty of determining clinically effective doses, all complicate the task of attempting to correlate quantitatively the results of biochemical studies of receptors in cell-free systems with results of clinical studies.

Many drugs that are used primarily in the treatment of schizophrenia have also been shown to be effective agents in the treatment of depression. Thus, the antipsychotic phenothiazines thioridazine⁴⁸, chlorpromazine⁴⁹⁻⁵¹ and perphenazine⁵² were found in several studies to be as effective as conventional tricyclic antidepressant drugs in the treatment of certain forms of depression, particularly in alleviating depressed mood, somatic concerns, anxiety and psychomotor agitation. The antipsychotic thioxanthene, thiothixene, has also been judged to be highly effective in the treatment of depression⁵³⁻⁵⁵. It is therefore pertinent that diverse antipsychotic compounds are potent inhibitors of histamine-sensitive adenylate cyclase as measured in homogenates of guinea pig hippocampus (Table 3). Thioridazine and thiothixene were amongst the most potent inhibitors of histamine-sensitive adenylate cyclase, with inhibition constants of 0.011 μ M and 0.022 μ M, respectively. However, the ability to inhibit histamine-sensitive adenylate cyclase was not a property of all phenothiazines, for diethazine, a phenothiazine devoid of antipsychotic properties, had little effect on the enzyme. Many of the compounds listed in Table 3 that are potent inhibitors of histamine-sensitive adenylate cyclase have not been tested clinically as antidepressant agents.

Many antipsychotic drugs effective in the treatment of schizophrenia are potent inhibitors of histamine-sensitive adenylate cyclase activity. The tricyclic antidepressant drugs that are known to be ineffective in the treatment of schizophrenia¹ are also inhibitors of this enzyme. The spectra of inhibition constants for these two groups of compounds fall within a similar range. It therefore seems unlikely that the therapeutic effects of the antipsychotic drugs in the treatment of schizophrenia are a consequence of their ability to block this enzyme. However, as both the tricyclic antidepressant drugs and many antipsychotic drugs are known to be effective in the treatment of depression, the

Table 2 Calculated inhibition constants (K_i) of antidepressant drugs for histamine-sensitive adenylate cyclase activity in homogenates of guinea pig neocortex

Drug	K_a/K_i	K_i (μ M)
Dibenzazepine		
Chlorimipramine (0.6 μ M)	15.0	0.043
Imipramine (2 μ M)	13.3	0.16
Desmethylinipramine (5 μ M)	16.7	0.32
Dibenzocycloheptadiene		
Amitriptyline (0.8 μ M)	14.3	0.060
Nortriptyline (5 μ M)	18.9	0.28
Protriptyline (6 μ M)	21.1	0.30
Dibenzoxepine		
Doxepin (1.5 μ M)	9.0	0.19
N-substituted cycloalkylindole		
Ipindole (6 μ M)	37.1	0.17
Tetracyclic piperazine		
Mianserin (0.8 μ M)	12.2	0.071

Data from experiments (of the type shown in Fig. 1) in which the concentration of histamine was varied, and the concentration of antidepressant drug held constant. For each drug, the inhibition constant (K_i) was calculated as described in the legend to Table 1a. The concentration of antidepressant drug used in each experiment is shown in parentheses. At the concentrations used in these experiments, the antidepressant drugs had little or no effect on adenylate cyclase activity measured in the absence of added histamine.

Table 3 Calculated inhibition constants (K_i) of several antipsychotic drugs and related compounds for histamine-sensitive adenylate cyclase activity in homogenates of guinea pig hippocampus

Drug	IC ₅₀ (μ M)	K_i (μ M)
Phenothiazine		
Thioridazine	0.15	0.011
Chlorpromazine	0.55	0.041
Promazine	1.1	0.081
Perphenazine	0.80	0.059
Fluphenazine	0.95	0.070
Promethazine	0.40	0.030
Diethazine	N.S.*	—
Thioxanthene		
Thiothixene	0.30	0.022
Dibenzodiazepine		
Clozapine	2.3	0.17
Butyrophenone		
Haloperidol	1.1	0.081
Indolone		
Molindone	N.S.*	—

Data from experiments (of the type shown in Fig. 3) in which the concentration of histamine was held constant at 100 μ M, and the concentration of antipsychotic drug or related compound was varied. For each drug, the inhibition constant (K_i) was calculated, assuming competitive inhibition, as described in the legend to Table 1b. Basal adenylate cyclase activity was not altered over the range of concentrations of drugs used to determine the IC₅₀ values.

*Not significant (that is, < 25% inhibition observed at 100 μ M test substance).

effects of these compounds on brain histamine-sensitive adenylate cyclase are consistent with the hypothesis that certain of their therapeutic effects are a consequence of their ability to block histamine receptors in the brain.

Promethazine (Table 3) and cyproheptadine⁵⁶ are two other drugs that inhibit histamine-sensitive adenylate cyclase. They are inactive in many of the laboratory screening procedures which use animal behavioural models for depression⁵⁷, and consequently have not undergone clinical trials as antidepressant agents. If the therapeutic effects of the antidepressant drugs are a consequence of their ability to block histamine receptors in the brain, one might predict that promethazine and cyproheptadine would also possess clinical antidepressant properties. It seems unlikely, however, that every compound capable of inhibiting histamine-sensitive adenylate cyclase would be useful in the treatment of depression. This can be illustrated in the case of two compounds, lysergic acid diethylamide (LSD) and fluphenazine. LSD has been shown to be a competitive inhibitor of histamine-sensitive adenylate cyclase⁵⁸. However, the inhibition constant for LSD (1 μ M) is about 20-fold greater than the inhibition constant for chlorimipramine (0.055 μ M). Furthermore, the hallucinogenic properties of LSD, which are evident at extremely low doses, and which may be a consequence of the interactions of LSD with 5-HT^{58,59} or dopamine⁶⁰ receptors, would preclude its clinical use in the treatment of depression. Similarly, the low doses of the antipsychotic drug fluphenazine used clinically in the treatment of schizophrenia may be sufficient to cause *in vivo* blockade of brain dopamine-sensitive adenylate cyclase (K_i , 0.005 μ M)⁶¹, but not of brain histamine-sensitive adenylate cyclase (K_i , 0.070 μ M). Thus, potential antidepressant properties of fluphenazine may not be manifested at the dose rates in present clinical use. Many tricyclic antidepressant drugs have inhibition constants for brain histamine-sensitive adenylate cyclase in a range similar to that of fluphenazine; however, the much higher doses of the tricyclic antidepressant drugs used clinically in the treatment of depression are probably sufficient, as discussed above, to cause *in vivo* blockade of this enzyme.

Effects of inhibitors of MAO and biogenic amine reuptake

Two other classes of drugs were examined for possible effects on histamine-sensitive adenylate cyclase activity in homogenates of

guinea pig hippocampus. In these experiments, the concentration of test substance was varied while that of histamine was held constant at 100 μ M. One class of drugs examined included three monoamine oxidase (MAO) inhibitors (pargyline, isocarboxazid, tranylcypromine) which have been proven effective in the treatment of depression. At concentrations up to 10⁻⁴ M none of these compounds significantly influenced basal or histamine-stimulated adenylate cyclase activity. Indeed, the marked differences in clinical effects between these antidepressant compounds and the tricyclic antidepressant drugs⁶² suggest that the properties of each class of drugs result from distinct molecular mechanisms of action.

A second class of drugs examined were certain compounds which inhibit the reuptake of monoamines into neurones. Thus, if the therapeutic effects of the tricyclic antidepressant drugs were a direct consequence of their ability to block NA and 5-HT reuptake processes, one might expect that other inhibitors of the reuptake process would also be effective antidepressant agents. In certain cases, this has not been observed. Cocaine is an inhibitor of NA⁶³ and 5-HT⁶⁴ reuptake in brain slices, and has euphoriant effects in normal individuals. However, its clinical effects differ markedly from those of the tricyclic antidepressants: cocaine administered orally to depressed patients does not influence their depressive symptomatology, while administered intravenously it produces marked dysphoria⁶⁵. Thus, cocaine clearly does not possess antidepressant properties. It is therefore of interest that cocaine, at concentrations up to 10⁻⁴ M, has no effect on basal or histamine-stimulated adenylate cyclase activity. FG4963 (ref. 66) and fluoxetine⁶⁷ are extremely potent and highly selective 5-HT reuptake inhibitors. For this reason, FG4963 underwent clinical trials as a potential antidepressant drug, but was found to be devoid of antidepressant activity⁶⁸. Both FG4963 and fluoxetine were found in the present study to be much weaker than any of the antidepressant drugs tested as inhibitors of histamine-sensitive adenylate cyclase. The concentrations of FG4963 and fluoxetine required for half-maximal inhibition of histamine-sensitive adenylate cyclase activity were 12 μ M and 21 μ M, corresponding to inhibition constants of 0.89 μ M and 1.6 μ M, respectively. On this basis, one might predict that fluoxetine will also prove ineffective as an antidepressant agent.

Possible consequences of drug action on histaminergic systems

The data presented indicate that a large number of drugs of chemically diverse structures, all of which possess clinical antidepressant properties, are also potent inhibitors of histamine-sensitive adenylate cyclase in preparations from guinea pig hippocampus and neocortex. While each of these antidepressant drugs has many other pharmacological actions, members of this group do not share a known common site of action on any other molecular process in neuronal tissue. This suggests that blockade of histamine-sensitive adenylate cyclase may contribute to the antidepressant actions of these compounds. One corollary of this observation is that tests of the ability of compounds to inhibit this enzyme may provide a sensitive *in vitro* screening procedure in the search for new antidepressant drugs. We do not mean to imply, however, that all of the therapeutic effects of the antidepressant drugs result from their actions on this enzyme, nor that all of the manifestations of clinical depression are attributable to an abnormality of histaminergic systems in the brain. Indeed, any attempt to account for all of the complex clinical manifestations of depression by an abnormality in a single neurotransmitter system in the brain would probably represent a gross oversimplification.

Although antidepressant drugs would be expected to affect brain histamine-sensitive adenylate cyclase (and monoamine reuptake systems) within a short time after their administration, it is well known that these drugs require at least 10 d before their therapeutic effects become manifest. Recently, alterations in the sensitivity of NA^{69,70} and 5-HT⁷¹ receptors in brain have been found after chronic administration of antidepressant drugs, which correlate temporally with their therapeutic actions. These alterations in receptor sensitivity were not a consequence of the

effects of the antidepressant drugs on monoamine reuptake systems, since these changes were also induced by iprindole, an antidepressant drug which does not affect the reuptake process. It seems possible that the acute blockade of histamine receptors in brain by antidepressant drugs might result in decreased cyclic AMP levels in the postsynaptic cells, as well as in alterations in the functional activity of associated neuronal networks in the cortex and limbic system. Such changes might then affect transcriptional events in the nuclei of those neurones containing the histamine-sensitive adenylate cyclase, as well as in the nuclei of other functionally connected neurones, leading to alterations in receptor sensitivity. By such a theoretical mechanism, an immediate biochemical effect of the antidepressant drugs on histamine-sensitive adenylate cyclase might induce other delayed biochemical sequelae; these secondary biochemical events might then be responsible for, and correlate temporally with, the onset of their therapeutic antidepressant actions.

Prospects for the future

The fact that so many of the psychoactive drugs affect several neurotransmitter systems in the brain makes the elucidation of the mechanism of action of these drugs difficult, and makes analyses of the relative roles of neurotransmitter systems in psychiatric disorders even more difficult. However, it may be possible to alter specifically the activity of the histaminergic system in brain and thereby evaluate its possible role in depression. For example, the specific H₂-receptor antagonists metiamide and cimetidine^{72,73} have little effect on other biogenic amine receptors. These drugs, which are used clinically in the treatment of peptic ulcers, do not cross the blood-brain barrier, and therefore would not be expected to possess antidepressant properties. Perhaps such drugs could be chemically modified so as to cross the blood-brain barrier and still retain their high degree of specificity as H₂-receptor antagonists, for they would then be worthy of clinical trial as antidepressant agents. Conversely, histidine administration to animals increases levels of brain histamine⁷⁴, and may increase the functional activity of histaminergic systems in the brain. It would be of interest to determine if administration of large doses of histidine might precipitate or worsen depressive symptoms in certain groups of patients with affective disorders. Clearly, further biochemical, physiological and pharmacological studies on the histaminergic systems in brain, and the possible relationship of these systems to depression and to the mechanisms of antidepressant drug action, are worthwhile areas for future investigation.

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Evolution of homeothermy in mammals

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We propose that mammalian homeothermy was acquired in two steps. The first step enabled mammals to invade a nocturnal niche without an increase in resting metabolic rate. The second step enabled them to invade a diurnal niche and involved the acquisition of higher body temperatures and metabolic rates.

THE acquisition of a constant body temperature was a major event in the evolution of mammals. We have developed a hypothesis to explain how this occurred; it is based on a review of

the fossil record and a comparison of the energetics of insectivores, monotremes and marsupials.

Hypothesis

Our hypothesis is as follows. Homeothermy enabled the first mammals to invade a temporally wide nocturnal niche that had previously not been exploited fully by small insectivores. The body temperature of these first homeotherms was about 10 °C lower than the 38°-40 °C found in most present day mammals. These first mammals could maintain their lower constant body temperature without the high resting metabolic rate typical of most living mammals. The loss of visual information in a

nocturnal niche was compensated for by better developed senses of hearing and smell and a larger brain developed for processing this additional information. The combination of a low body temperature and a low metabolic rate restricted the first mammals to their nocturnal niche. At various times, small nocturnal mammals acquired higher metabolic rates, enabling them to regulate their body temperature at higher levels and to invade a diurnal niche. However, some living mammals are descended from forms that have remained in the nocturnal niche since it was first occupied successfully by mammals. They have retained their low body temperature and low reptilian-like metabolic rate, because it enables them to survive on much less energy.

Homeothermy

It seems reasonable to assume that the ability to maintain a constant body temperature was necessary in order fully to exploit a temporally wide nocturnal niche. Vertebrates can maintain a highly coordinated, finely tuned locomotor system over only a relatively narrow range of body temperatures (perhaps a maximum of 15 °C). Diurnal reptiles can search within a wide mosaic of ambient temperatures (shade, sun, burrows and so on) to maintain their body temperature within optimal limits and they can remain active for relatively long periods¹. However, when the Sun sets, the diversity of available ambient temperatures (particularly radiation temperatures) decreases considerably. A small animal that could use its metabolic heat production and insulation to maintain a constant body temperature could remain active and forage for much longer periods in a nocturnal environment than an animal that had to rely on the diversity of available ambient temperatures.

Evidence from the fossil record suggests that the first mammals (Fig. 1) exploited a vacant nocturnal niche. The major differences between the advanced mammal-like reptiles and the earliest mammals are a four- to fivefold increase in the relative size of the brain^{2,3} and a decrease in body size by an order of magnitude (to 30–40 g)⁴. The increase in brain size seems to be due chiefly to improved senses of smell and hearing. Jerison³ has argued that the improvement in these senses compensated for a loss of visual sensory information as mammals invaded a nocturnal niche. The postcranial skeleton of the first mammals suggests that they lived in the same habitat where many small insectivores live today. It was adapted for moving on spatially complex, uneven surfaces such as would be encountered at the interface between arboreal and terrestrial habitats⁴.

At what temperature should these first mammals have regulated their bodies in order to invade a nocturnal niche? There is an obvious energetic advantage for a homeotherm to set its body temperature as low as possible.

The metabolic rate of a resting animal increases two- to threefold with each 10 °C increase in temperature (Q_{10} of 2–3). Thus as body temperature increases, more time will have to be spent in acquiring and processing food. However, there are also good reasons why small homeotherms should regulate their body temperatures several degrees above the highest ambient temperature which they are likely to encounter regularly in nature. Once ambient temperature exceeds body temperature, a homeotherm must resort to evaporative cooling to maintain a constant temperature. It is impossible for small vertebrates (less than 1 kg) to sustain the high rates of evaporation necessary to maintain their body temperature even 2° or 3° below ambient temperature for more than a few hours, and the problem becomes more acute the smaller the animal⁵. The large surface to volume ratio of small animals results in a rapid inward flow of heat; large amounts of water have to be evaporated to keep cool, and animals would have to drink almost continuously to avoid lethal levels of dehydration. These problems can be avoided if body temperature is regulated at a level a few degrees above ambient temperature because this facilitates the control of outward flow of heat simply by varying insulation. From these considerations, it seems that an optimal body temperature for small homeotherms would be just high enough to avoid the necessity for prolonged evaporative cooling. A nocturnal fossorial animal could operate very well with a constant body temperature of 25°–30 °C, while a diurnal

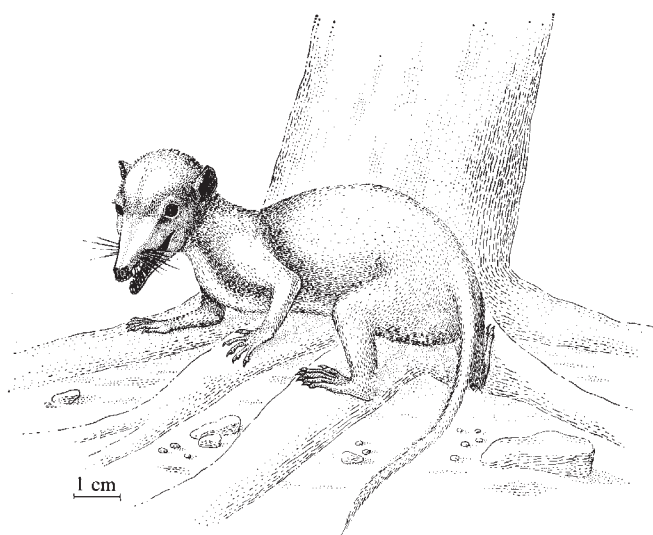


Fig. 1 Reconstruction of one of the earliest known mammals (*Megazostrodon rudnerae*) from the late Triassic, about 200 Myr ago.

animal which encountered solar radiation would need its temperature closer to 40 °C.

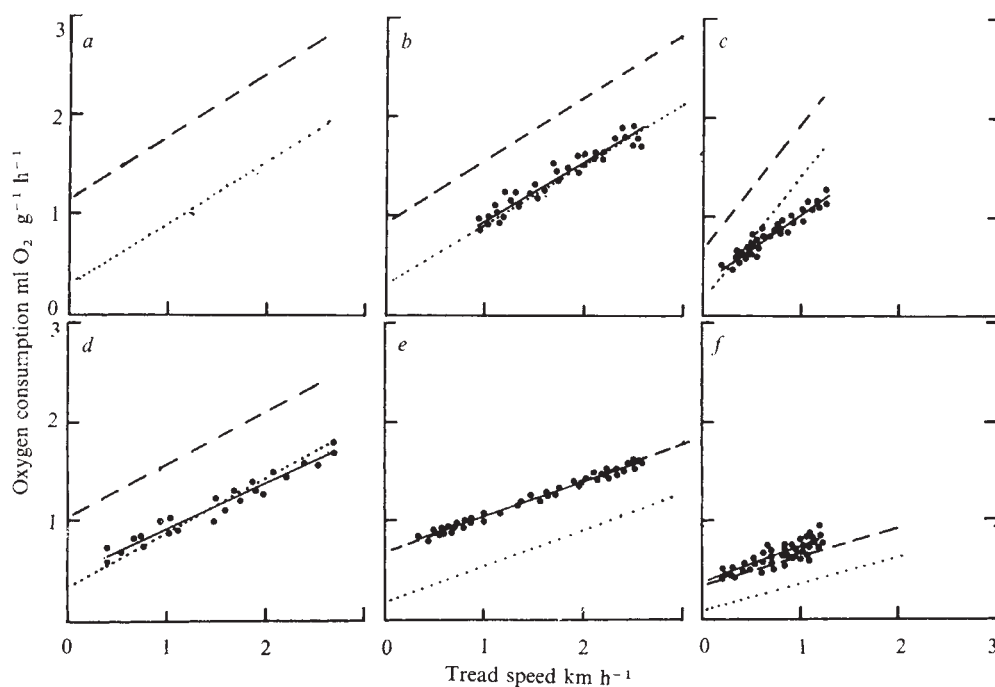
It is frequently argued that it is advantageous for homeotherms to regulate their body at the highest temperature compatible with life, because this would enable them to develop more metabolic power and to move faster. The assumption implicit in this argument is that rates of metabolism and rates of muscular contraction are limited by temperature. The evidence, however, does not support this assumption. Brett⁶ has pointed out that the resting metabolic rates of Antarctic fish acclimated to 0°–5 °C are approximately the same as those of tropical fish acclimated to 25°–30 °C. During acclimation temperature effects on the rate of metabolism are compensated for by changes in the enzymes which catalyse the reaction. The speed of muscular contraction also seems to be limited by factors other than temperature. The intrinsic contraction velocity of equivalent muscles of different sized animals is proportional to (body weight)^{0.125} for structural reasons⁷. If muscle contraction velocities were limited by temperature, one would expect that the optimum body temperature of homeotherms would fall as body size increased. Instead, it is the intrinsic speed of muscular contraction which decreases as animals get larger. Equivalent muscles of a 30-g mouse contract about three times as rapidly as those of a 500-kg horse. If temperature limited speed of contraction, the horse could have the same contraction speed as the mouse with a body temperature about 10 °C lower (assuming a Q_{10} of 3). There is also evidence that animals of the same size with very different body temperatures can reach nearly the same top running speeds. For example, we found that one of our insectivores which had a body temperature of 28 °C ran faster than our white rats which had a body temperature of 38 °C. In nature geckos can move as fast as small mammals when capturing an insect, although their body temperature may be 10°–15° lower. The conception that speed of contraction increases with increasing body temperature probably arises from our experience in dealing with reptiles which are sluggish when they are cold. There is no question that their rate of contraction increases with increasing temperature as they warm to some 'optimal' body temperature. This optimal temperature, however, may be as low as 20 °C. If the reptile is warmed further to higher body temperatures, then the speed of contraction and power output of the muscles decrease. There are good biochemical reasons for such optimal temperatures with a decrease in activity on either side⁸.

If the first mammals were nocturnal, they could have maintained a constant body temperature of 30° without the three- to fivefold jump in resting metabolic rate (after normalisation for

temperature and size) which is generally assumed to be a prerequisite for homeothermy⁹⁻¹¹. They would seldom, if ever, have needed to encounter a gradient between body and ambient temperature exceeding 10 °C. However, small diurnal homeotherms, which would have needed a higher body temperature of about 40 °C, would probably have required the higher metabolic rates to maintain this temperature against the steeper gradients that they would have encountered at night. The fossil record indicates that for a period of about 120 Myr (during Jurassic and Cretaceous times) most mammals remained in a nocturnal insectivorous niche. The major radiation of mammals occurred at the beginning of the Tertiary. During the late Cretaceous, there were about five families of placentals, three families of marsupials and six families of multituberculates in existence. At the end of the Cretaceous, most marsupials became extinct. Before the end of the Palaeocene, at least 40 families of mammals were in existence¹². Most of the new groups seem to have been diurnal forms, whereas most families of insectivores seem to have remained in the more conservative nocturnal insectivore niche. The invasion of a diurnal niche probably occurred independently several times, beginning in the Cretaceous with the multituberculates and some

which appear to have remained in the nocturnal insectivorous niche. The Tenrecidae have been isolated from the mainstream of mammalian evolution for about 75 Myr. They retain many primitive morphological characteristics: molar structure; a cloaca; and testes which do not descend during maturation. Their ecology, behaviour and observed body temperature on Madagascar today are similar to those we have proposed for the first mammals. Eisenberg and Gould¹⁴ have reported in their monograph on tenrecs that most of the Tenrecidae live in bush or undergrowth associated with forests. They dig burrows where they seek shelter from the heat of the day. They are active during the night when they forage or dig for invertebrates (earthworms, insects and their larvae). Their auditory, chemical and tactile senses are well developed and are used for communication, foraging and defense. When body and air temperatures of *Setifer setosus* were taken at the time the animals emerged from their burrows and commenced foraging, body temperature averaged 29.5 °C (range of 28.5–30.5 °C) and ambient temperature varied between 21° and 24 °C. Other species of Tenrecidae seem to have similar low body temperatures, a few degrees above the highest temperatures encountered in nature.

Fig. 2 Relationships between rate of oxygen consumption and speed predicted for a mammal (dashed line) and a lizard (dotted line) (details of the calculations in ref. 20) are plotted in (a) for a hypothetical mammal and lizard each weighing 675 g and with a body temperature of 38 °C. Similar predicted relationships are also plotted in the other graphs for a mammal and a lizard of the same weight and body temperature as our experimental animals. b, tenrec, 675 g, 35.7 °C; c, setifer, 120 g, 28 °C; d, hedgehog, 10.5 kg, 37.5 °C; e, opossum, 27 kg, 36.2 °C; f, echidna, 50.4 kg, 31 °C. A comparison of our experimental data (solid line in each graph) with predicted relationships shows that the tenrec, setifer and hedgehog possess lizard-like energetics while the opossum and echidna possess mammal-like energetics. Slopes and y intercepts (calculated using the method of least squares) for these data together with the average body weight and body temperature during the experiments are as follows.



	Slope (ml O ₂ g ⁻¹ km ⁻¹)	y intercept (ml O ₂ g ⁻¹ h ⁻¹)	n	r	Weight (g)	T _{body} (°C)
Tenrec	0.58	0.39	35	0.93	675	35.7
Setifer	0.68	0.37	32	0.94	120	28.0
Hedgehog	0.46	0.48	24	0.91	1,050	37.5
Opossum	0.36	0.67	44	0.98	2,700	36.2
Echidna	0.30	0.35	43	0.69	5,040	31.0

of the marsupials. Kielan-Jaworowska¹³ has suggested that the modern monotremes were descended from the multituberculates, and thus the ancestors of these 'primitive' mammals may have been among the first to invade a diurnal niche.

Energetics

If the first mammals were nocturnal and if they possessed a low body temperature and low metabolic rate as we have proposed, we would expect that their descendants whose ancestors have remained in the nocturnal insectivore niche would have retained these energy saving characteristics. The Tenrecidae and the Erinaceidae are two families of insectivores descended from forms

The activity patterns, behaviour and body temperature of the Tenrecidae are consistent with our hypothesis, but what about their energetics? Do these insectivores have a low metabolic rate similar to that of reptiles? One should be able to answer this question by using the relationships developed by Dawson and Hulbert¹⁵ to distinguish between mammalian and reptilian energetics. If Tenrecidae possess reptilian-type energetics, their resting metabolic rate should be 1/3 to 1/4 the value predicted for a mammal by Kleiber's equation¹⁶ (after normalising the rates to a body temperature of 38 °C by assuming a Q_{10} of 2.5). If the lowest values for resting metabolic rate reported for *Tenrec ecaudatus*¹⁷ are treated in this manner, we obtain resting

metabolic rates less than 1/3 those predicted for a mammal, suggesting reptilian-type energetics. However, if the highest reported resting values are taken, the rates obtained are nearly 3/4 the predicted value. A similar situation exists for the resting metabolic measurements of hedgehogs¹⁸—it seems that the Ethiopian hedgehog has reptilian-type energetics and the European does not. Measurements of resting metabolic rates are notoriously variable, and the investigator must exert considerable judgement in deciding on a standard or basal level. Although resting measurements have proved extremely valuable in broad comparative studies such as those of Kleiber¹⁶ and Dawson and Hulbert¹⁵, it is much more difficult to use resting metabolic measurements of one or two species to decide whether or not they possess reptilian or mammalian-type energetics.

We can avoid much of the variability inherent in resting metabolic measurements by comparing energetics of exercising animals over a range of exercise intensities. The metabolic rate of a mammal exercising at a given intensity is much more reproducible than its resting metabolic rate. Bakker¹⁹ has used this approach to compare the energetics of lizards and mammals over a range of running speeds. He found the rates of oxygen consumption of a lizard and a mammal of the same weight increased at about the same rate with increasing speed, the metabolic rate of the lizard was lower by a nearly constant amount at any speed (Fig. 2). This constant difference was approximately the same as the difference which Dawson and Hulbert¹⁵ would have predicted between a resting lizard and a resting mammal after normalising the rates to the same body temperature. Therefore we decided to use measurements of the relationships between rates of oxygen consumption and running speeds, rather than resting rates, to distinguish between reptilian and mammalian energetics.

We selected three insectivores (two Tenrecidae—tenrecs and setifers, and one Erinaceidae—the European hedgehog), one marsupial (opossum) and one monotreme (echidna) to study their energetics. We had data relating rates of oxygen consumption and speed for four various lizards¹⁹ and mammals²⁰ available from previous studies in our laboratory. We trained three European hedgehogs (*Erinaceus europaeus*, average weight 1.05 kg); three tenrecs (*Tenrec ecaudatus*, 695 g); three setifers (*Setifer setosis*, 120 g); two opossums (*Didelphis virginiana*, 2.70 kg); and two echidnas (*Tachyglossus aculeatus*, 5.04 kg) to run on treadmills while we measured the rate at which they consumed oxygen ($\dot{V}O_2$). The techniques for measurement of oxygen consumption have been described elsewhere²¹. The accuracy of our system was better than 4%. Animals were trained for a minimum of 4–5 months and all measurements were steady state (defined as varying less than 5% over a 30 min run at any given speed). We also measured rectal or cloacal temperature during the run.

$\dot{V}O_2$ increased nearly linearly with speed in all of these species (Fig. 2). Slopes and y intercepts of these linear relationships were determined using the method of least squares and are given in the legend to Fig. 2. We have plotted two lines on the graph for each species in addition to the solid line representing the linear regression of the data. The dashed line is the predicted relationship for a mammal of the same body weight and temperature as our experimental animal, and the dotted line is the predicted relationship for a lizard²⁰. Comparison of our experimental data with the predicted relationships enables us to decide whether a species possesses reptilian or mammalian-type energetics. If the graphs are used in this way, the three species of insectivores (tenrecs, setifers and hedgehogs) seem to have reptilian-type energetics, while the marsupial (opossum) and monotreme (echidna) seem to have mammalian-type.

The predictions of our hypothesis for energetics seem to be substantiated. Insectivores, which have remained in the nocturnal niche, have retained reptilian-type energetics. Monotremes and marsupials, whose ancestors were at one time diurnal, have acquired typical mammalian-type energetics. Both echidnas and opossums are now nocturnal and regulate their body temperatures while resting at night at about 30°–35°C. However, they apparently have not been able to re-acquire reptilian-type energetics.

Some consequences of our hypothesis

We have proposed that mammalian homeothermy was an adaptation which opened up a vacant nocturnal niche for small insectivores. Others have proposed that the mammal-like reptiles were homeotherms long before the first mammals appeared^{10,11}. These proposals are mutually exclusive, therefore it seems worthwhile to review the evidence used to argue that mammal-like reptiles were homeotherms.

Ricqlès¹¹ has used similarities in bone histology between modern mammals and mammal-like reptiles to argue that mammal-like reptiles were homeotherms. Bones from therapsids and modern mammals both have well vascularised fibrolamellar bone containing primary osteons and highly developed Haversian substitution systems. These two features are not found in most modern reptiles which have been investigated. Ricqlès argues that a high degree of vascularisation and Haversian substitution systems imply rapid growth rates, high rates of freeing or fixing phosphocalcic salts and a high metabolic rate. He therefore concludes that the therapsids possessed high metabolic and growth rates. He then argues that high metabolic and growth rates are evidence of homeothermy. Ricqlès, however, points out that ectothermic reptiles which have high growth rates during early life and are capable of continuous muscular activity over long periods (for example, sea turtles) have "fair" vascularisation of some bones and extensive Haversian substitution systems. Additionally, small mammals and passerine birds lack the "typical" mammalian bone histology. Therefore, Ricqlès' data clearly suggest that bone histology reflects the level of overall activity and growth rates, but he is unable to establish a clear link between bone histology and homeothermy.

Bakker¹⁰ has argued that mammal-like reptiles were homeotherms on the basis of therapsid predator/prey ratios which he has measured in the Late Permian and Early Triassic fossil deposits. He argues that predator/prey ratios of ectothermic communities are an order of magnitude greater than the ratio of an endothermic community. We agree with Bakker that the predator/prey ratio observed for therapsids suggests a high rate of activity. The efficient masticatory apparatus of therapsids rivals that of modern mammals in the complexity of the tooth crown pattern and jaw movement²² and this also suggests high rates of processing food. It might well have been possible to develop high rates of activity without homeothermy, just as we have shown that it is possible to be a homeotherm with a low resting metabolic rate. We feel that neither rate of activity nor resting metabolic rate are proof of homeothermy and that the mammal-like reptiles may well have been ectotherms.

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letters to nature

γ -Ray burst observed at balloon altitude

SINCE the γ -ray burst was discovered in 1973, approximately 50 events have been observed using artificial satellites^{1,2}. In addition, several bursts of smaller size have been found using balloon-borne detectors³⁻⁶ with large sensitive areas. No burst has yet been located on the celestial sphere, with an adequate precision to associate it with an astronomical object. To determine the precise position of a γ -ray burst which had not been predicted to occur, the detector must have a wide field of view and the capability of precise location of the source. A rotating cross-modulation-collimator (RCMC) proposed⁷ as a device to fulfill these apparently conflicting requirements was used in the series of balloon observations reported here. A small γ -ray burst was found during ~ 150 h of observations and its celestial position was determined with a precision of $\sim 0.3^\circ$.

Two modulation collimators were installed in the balloon gondola so that the fields of view of the collimators were pointed towards the zenith, and the directions of the collimator transmission bands projected on the celestial sphere were perpendicular to each other. Scintillation counters of NaI(Tl) of $5''$ in diameter were installed underneath the two collimators. An additional monitor detector, which consisted of a conventional collimator and a NaI(Tl) counter of $4''$ in diameter, produced the time profile of the unmodulated X-ray flux. The top view of the detector is shown in Fig. 1. By rotating this device, the modulation pattern corresponds to the position of the burst source. The pitch of the modulation peaks is inversely proportional to the projected angular distance

Fig. 1 Top view of the detector.

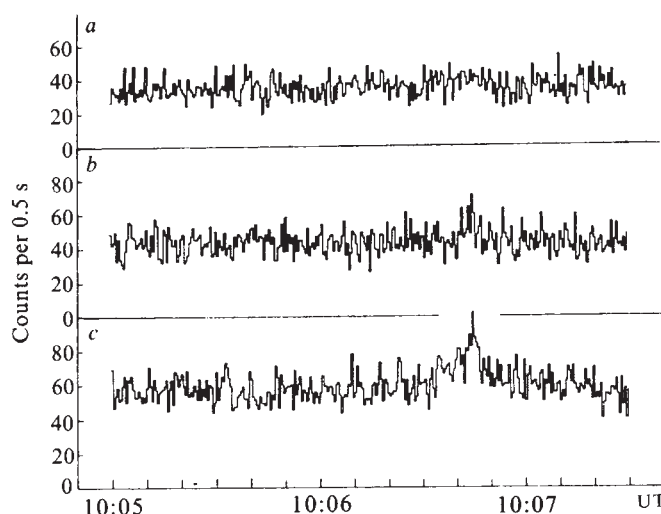
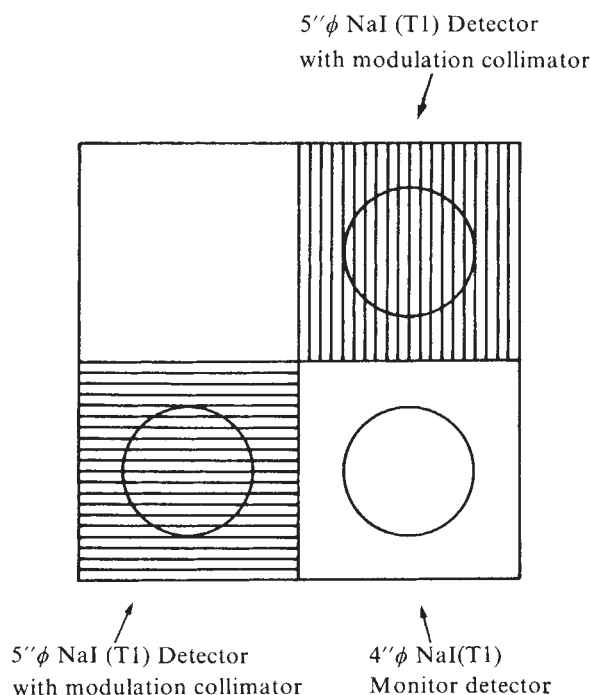


Fig. 2 Time profile of the count-rate 23 September 1975: *a*, and *b*, observed by the detector with the different modulation collimator as shown in Fig. 1; *c*, observed by the detector without the collimator.

of the burst source from the spin axis on the direction of collimator wires. Thus the observed patterns through the two collimators provide us with information on the burst source. Each bi-grid modulation collimator was constructed with grids of tungsten rods of 1 mm in diameter, with 1 mm spacing. The separation of the two grids was 5 cm, providing the transmission band-spacing of 2.29° . The field of view of each counter was approximately $100^\circ \times 100^\circ$ FWHM. The X-ray energy band from 20 to 200 keV was divided into four channels, 20–40, 40–60, 60–100 and 100–200 keV, respectively. To avoid the instrumental effects, electronic circuits, power supplies and high voltage supplies for each of three detector systems were independent.

Three identical balloon payloads were prepared. Three balloons were launched from Sanriku Balloon Center on 23, 24 September and 4 October 1975. They reached the ceiling altitude of approximately 5 mbar. The gondolas were rotated around the vertical line at a rate of 2 r.p.m. The observations were performed for ~ 55 h, 65 h and 25 h respectively, the total observational time being about 150 h. Subtracting the overlapping time of the first two flights, we monitored the γ -ray burst for ~ 120 h. A significant increase of the count-rate was found at about 10 h 06 min 40 s UT, 23 September as shown in Fig. 2. The event started at about 10 h 06 min 31 s UT, reached the maximum at 06 min 42 s and disappeared by 07 min 00 s. The signal-to-noise ratio of the event observed by the monitor counter was $\sim 11\sigma$. The energy spectrum of the burst observed by the monitor counter is shown in Fig. 3. The atmospheric absorption is corrected and the intensity given is the average for the period from 06 min 39 s to 44 s. When the event was observed, the position of the balloon was at $144^\circ 15' 44''$ E, $38^\circ 36' 36''$ N. The L -value of the location is 1.30.

The event is unlikely to be of solar-terrestrial origin because (1) magnetic latitude of the balloon location was comparatively low, as indicated by the small value of L ; (2) the event occurred

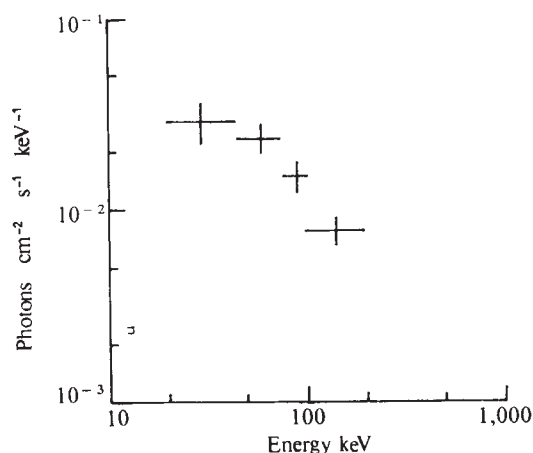


Fig. 3 The energy spectrum of the event.

more than 2 h after the sunset; (3) the solar-terrestrial environment was quiet⁸ when the event was observed; (4) as the X-ray flux was modulated by the collimator it indicated that the source is not as extended as expected for the terrestrial phenomena; (5) the energy spectrum is similar to that of the γ -ray burst. Hence, we concluded that the event is a small γ -ray burst.

The position of the spin-axis of the gondola in the projected field of view of the collimator on the celestial sphere was determined using Cyg X-1 as the reference. The offset angle of the spin axis was $0.4^\circ \pm 0.1^\circ$ from the direction of the maximum transmission of the modulation collimators. To determine the burst position, a correlation map⁹ of the celestial sphere

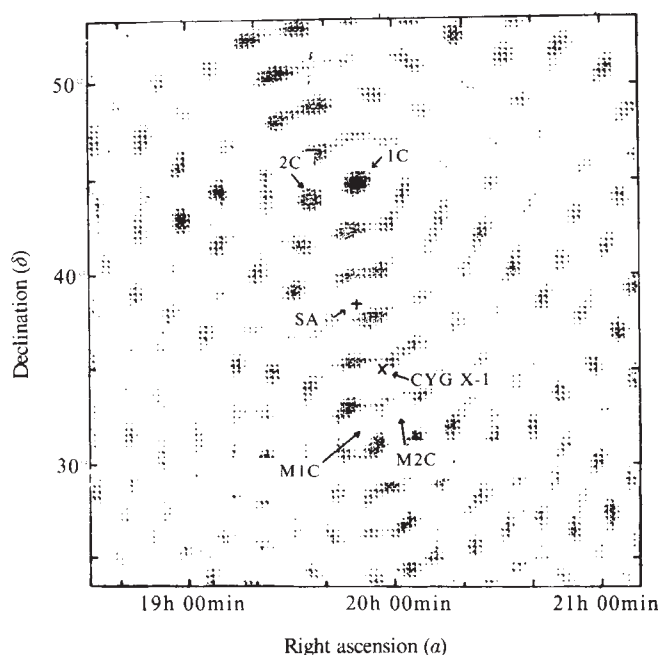


Fig. 4 Correlation map for locating the celestial position of the burst source. The blackness of each point shows the degree of correlation between expected modulation of count-rates for each celestial position and observed modulation. Mirror images of the candidate positions with respect to the spin axis are indicated. The sign of the mirror image is negative, as expected where the offset of the spin axis from the transmission band is about 1/4 of the band spacing. 1C, first candidate; 2C, second candidate; MIC, mirror image of first candidate; M2C, mirror image of second candidate; SA, spin axis.

was produced using the count-rates of the modulation collimator counters normalised by the output of the monitor counter. The 'offset' of spin axis from the direction of maximum transmission resolves the redundancy inherent to the modulation collimator in locating the position. The position of the burst can thus be determined if the effect of the counting fluctuations is ignored. To reduce these fluctuations, we combined the data from the two modulation collimator detectors considering the 90° difference of the rotational phase of the two collimators. The details of analysis will be published elsewhere.

The correlation map obtained from the above analysis is shown in Fig. 4. The most likely position of the burst source is at $\alpha = 19^{\text{h}} 49^{\text{m}} \pm 2^{\text{m}}$, $\delta = 44^\circ 05' \pm 15'$ (1950) approximately 10° north north-west of Cyg X-1. The second candidate position, $\alpha = 19^{\text{h}} 35^{\text{m}} \pm 2^{\text{m}}$, $\delta = 43^\circ 56' \pm 15'$ (1950) is also probable but with less likelihood, 50%, compared with the former. The mirror images of the candidates with respect to the spin axis are also shown. The sign of the mirror image is negative, as expected for the present case where the spin axis is offset by approximately 1/4 of the band spacing of the modulation collimator.

The burst occurred when Cyg X-1 which is suspected to be one of the burst sources¹ was in the field of view. Cyg X-1 is unlikely to be the source of the present event as the possibility is estimated to be 0.1%.

We conclude that we have located a γ -ray burst with a considerable precision for the first time using a single experimental device; the celestial positions of γ -ray bursts have previously been determined through the method of time of flight among two or more satellites.

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Io-phase motion and jovian decametre source locations

Io, Jupiter's innermost Galilean satellite, plays a major part in determining when jovian decametre storms are observed¹. Specifically, as a result of beaming of the radiation at a preferred angle to the local magnetic field, the most favourable satellite locations for the reception of emission are approximately 90° and $240^\circ \gamma_{10}$ (departure of Io from superior geocentric conjunction). Recently a second-order effect has been noted—a periodic displacement in the particular γ_{10} phase at which the reception probabilities are observed to peak²⁻⁴. The modulation has a peak-to-peak magnitude of 5° to 10° , a period of about 12 yr, and is most pronounced in the case of the jovian decametre storm sources Io-B and Io-C. (There is not yet general agreement as to the behaviour of Io-A.) The effect has been tentatively correlated with the $\pm 3.5^\circ$ variation in the jovicentric declination of the Earth, D_E ; however, it has also been considered³ that it may be a consequence of changes intrinsic to the jovian activity cycle. Here we interpret this phenomenon simply in terms of the latitudinal variations inherent in the Earth-Jupiter viewing geometry as defined by the 12-yr cycle in D_E . Furthermore, we show that the phase of the Io-B and Io-C displacements relative to D_E phase uniquely defines the hemisphere, north or south, from which the radiation associated with each source is beamed. The results agree with, and lend credence to, the source positions as inferred from polarimetry^{5,6} and more recently from considerations of planetary-limb shadowing⁷.

As working hypotheses we adopt three widely-held views regarding the jovian emission. We assume first that the radiation originates on either the north or south magnetic tube of flux which 'threads' Io (north or south IFT), second, that the emission takes place at the local electron gyrofrequency, ν_e , and third, that the Io-phase source morphology is a consequence of beaming of the radiation at some angle to the magnetic field. The first two assumptions constrain the source to lie at one or the other of two points in Jupiter's near-surface magnetosphere, resolution of this hemisphere ambiguity being provided by the evidence presented below.

We define the viewing geometry in terms of the angle (β) between **B**, the magnetic field vector in the source region, and

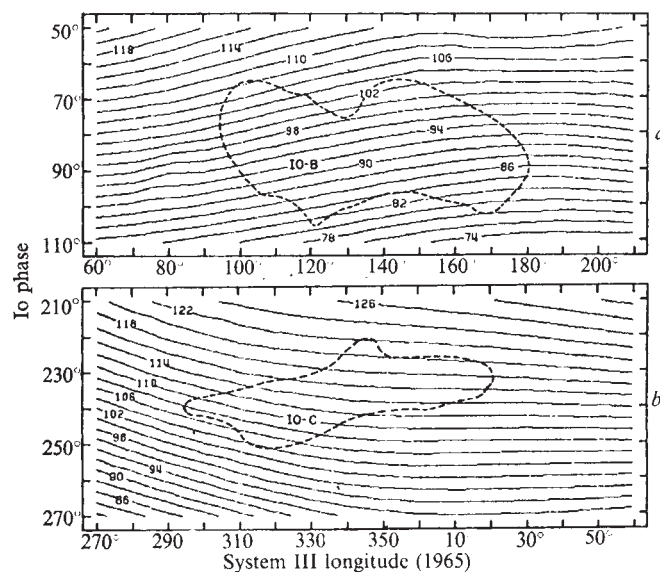


Fig. 1 The north- (a) and south- (b) IFT viewing geometries are shown in the $\lambda_{III}-\gamma_{10}$ plane for $D_E = 0^\circ$ and $\nu_e = 22$ MHz. The labelled contour lines show angle β in degrees. Dashed lines denote the approximate Io-B and Io-C source boundaries at 22 MHz.

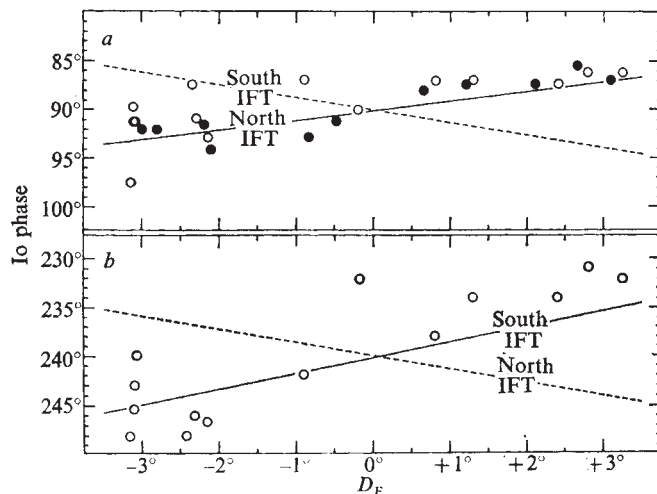


Fig. 2 The expected Io-phase behaviour of the Io-B (a) and Io-C (b) source centres is shown as a function of D_E . Solid and dashed lines are used to illustrate the north- and south-IFT cases individually. The empirically derived phase shifts ($\bullet$, ref. 2; $\circ$, ref. 3) are in close agreement with the expected behaviour of the north-IFT in the case of Io-B and the south-IFT in the case of Io-C.

$\mathbf{r}$, the Earth-directed radius vector; then ($\beta = \cos^{-1}(\mathbf{B} \cdot \mathbf{r})$). A model of the planetary magnetosphere derived from the *in situ* measurements of Pioneer 11 (ref. 8) is used to configure the near-surface field. Because this field is more complex than that of a simple, coaxial dipole, β is not only a function of γ_{10} , but also depends on ν_e (which specifies the source location), and on λ_{III} , the system (III) rotation phase of the planet. Through its dependence on $\mathbf{r}$, β is also a function of D_E .

In Fig. 1, contour lines labelled in degrees show the results of computing β over the $\lambda_{III}-\gamma_{10}$ coordinates of Io-B (north IFT only) and Io-C (south IFT only). For the cases shown, $D_E = 0^\circ$ and $\nu_e = 22$ MHz. Dashed lines denote the approximate source boundaries as they appear at 22 MHz. The value of β at the centre of each source, designated β_0 , is equal to about 90° for Io-B and 112° for Io-C. The corresponding value of β_0 for each opposite-hemisphere case (not shown) is about 92° for Io-B and 75° for Io-C (see ref. 7). Following assumption three, we take β_0 to represent the principal beaming angle of the radiation. The magnitude of the β contours is not as important in this treatment as the shifts in the contours due to changes in D_E , as derived below. Indeed, since the near-surface field has not been sampled directly, the values are probably inexact; however, the shifts themselves are reliable as they are primarily a function of the radius vector, $\mathbf{r}$.)

In order to investigate the effect of changes in D_E on the viewing geometry, β contours were calculated using $D_E = \pm 3.5^\circ$. For each source and IFT hemisphere, the resultant Io-phase shift of the β_0 contour ($\Delta\gamma_{10}$) was noted; as might be expected, the displacements were small. (For simplicity, because the shifts were slightly λ_{III} dependent, $\Delta\gamma_{10}$ was measured only at the λ_{III} coordinate of each source centre.) Measurement of the β_0 shift clearly allows us to predict the extent to which each source will be displaced in the γ_{10} coordinate as a consequence of changes in D_E . That is, because each source continues to beam radiation preferentially at β_0 , regardless of our vantage point in space, the expected Io phase of the source centre for a given D_E is simply the Io-phase coordinate of β_0 evaluated at that particular D_E . Thus, we obtain the theoretical source displacement in Io phase as a function of D_E .

The results of this analysis are shown in Fig. 2. For each source, both the north- and south-IFT geometries have been considered. It is apparent, after comparing the predicted behaviour with the empirically derived displacements, that the north IFT provides the best fit to the Io-B data and the south

IFT matches the Io-C data. The opposite-hemisphere geometries yield predictions which are clearly out of phase with the observations. Furthermore, within the uncertainty of the data, the present analysis properly accounts for the magnitude of the Io-phase variation in each source.

We conclude, therefore, (1) that the observed variation in Io phase is due solely to the latitudinal changes in Earth-Jupiter viewing geometry described by D_E and (2), that the Io-B (Io-C) source emission is beamed from northern (southern) magnetic latitudes. The latter is in agreement with source-location assignments based on the observation of predominantly right- (Io-B) and left- (Io-C) hand polarised emission and the assumption of strong magnetoionic mode coupling during propagation from the source region^{5,6}. A study which investigated the unequal visibility of the north- and south-IFT feet yielded the same conclusion⁷.

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Cooling of the Sun's photosphere coincident with increased sunspot activity

IN summer 1975, I began to monitor the spectroscopic temperature of integrated sunlight ('the Sun viewed as a star'). The first 1.5 yr of observations were taken at a time of minimum activity and the full disk temperature was apparently constant at $\pm 1-2$ K. However, 1977 produced a marked increase of activity with the onset of cycle no. 21. In response to this activity the Sun seems to have cooled by ~ 6 K. To the extent that the Sun is a black body, a 6 K change in temperature corresponds to a $\sim 0.5\%$ reduction in luminosity. The inverse relation between activity and temperature is a new discovery, and may prove helpful in explaining certain aspects of the Sun-Earth climate relationship.

The strengths of selected Fraunhofer lines depend critically on temperature, as indicated by the Saha-Boltzman formula. For thermal monitoring, a $3 \text{ \AA}$ spectral interval at $5,380 \text{ \AA}$ is observed using a double-pass high dispersion scanning spectrometer¹. Within this interval are three lines having different temperature dependence. A medium strength neutral iron line of low excitation (E.P. = 3.7 V), a weak high excitation line of carbon (E.P. = 7.7 V), and a line of singly ionised titanium (I.P. = 6.8 V, E.P. = 1.6 V). Holweger calculated the integrated-flux temperature sensitivity for these lines (personal communication). He found for Fe 5,379.6, $\Delta w/w = -0.00063 \text{ K}^{-1}$; for C 5,380.3, $\Delta w/w = +0.00123 \text{ K}^{-1}$; and for Ti(II) 5,381.0, $\Delta w/w = -0.000075 \text{ K}^{-1}$, where w is the equivalent width, that is, the total energy absorbed by the line from the continuum. Thus C 5,380 has a strong positive temperature dependence, Fe 5,379 a weak negative dependence, and Ti(II) 5,381 is virtually temperature insensitive. An independent empirical determination for C 5,380 by Hill *et al.*², using solar granulation, agrees with the calculated value. This C 5,380 line is of particular interest because of its superior temperature sensitivity and the fact it is formed within the same photospheric layers which give rise to the $5,300 \text{ \AA}$ continuous radiation. The Fe and Ti(II) lines originate higher in the solar atmosphere and may be subject to slight non-LTE effects.

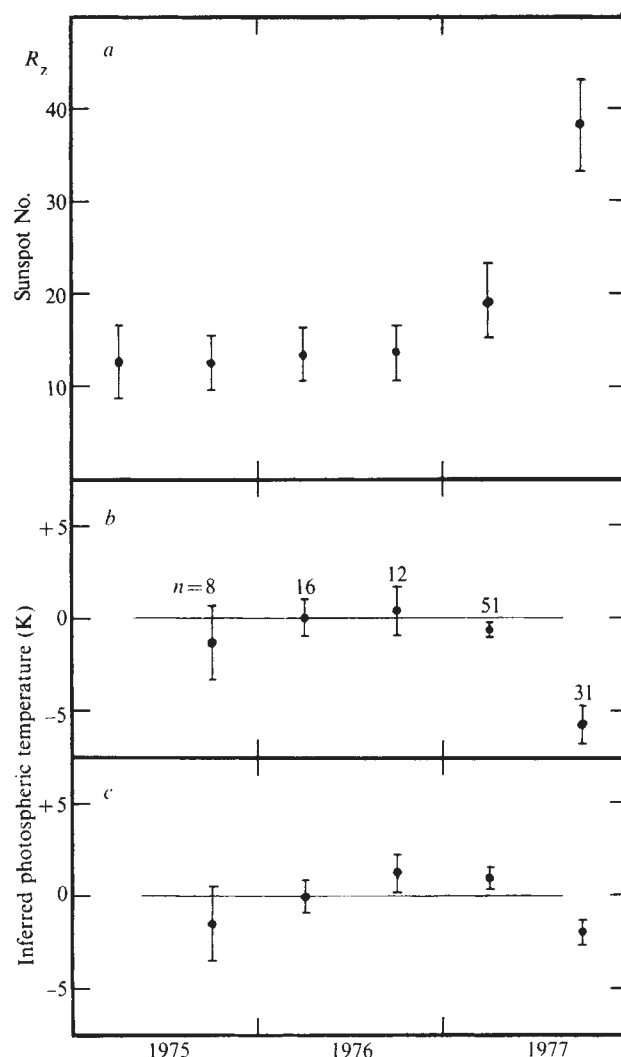


Fig. 1 *a*, Solar activity, as indicated by mean sunspot numbers R_z (Zurich sunspot number), compared with (*b*) and (*c*), the inferred photospheric temperature change in degrees Kelvin (K). Emphasis is placed on *b*, the C 5,380 data, with *c*, Fe 5,379 to be considered merely supportive. Each year is divided into two intervals: January to June and September to December, with July and August discounted because of water-vapour contamination. Zero point of the temperature scale is arbitrarily assigned to spring 1976. The error bars on the 1975-76 data are traceable to instrumental setup variability plus inadequate sampling (n = number of days). Both setup and sampling error are negligible in 1977, but now the facular fluctuation dominates because of new cycle activity.

Compared with other radiometric techniques for measuring the temperature change of stars, the spectroscopic method has the advantage that it is almost independent of terrestrial atmospheric absorption effects, the line strength being measured relative to the local continuum. Several sources of error do exist and they are traceable (1) to the variability of spectrograph resolution with the setup; (2) to the low frequency components of scintillation; (3) to slight residual water-vapour absorption near $5,380 \text{ \AA}$ found during the 'monsoon' months of July and August in Arizona, and (4) to white light faculae near the limb. The setup variability factor (1) arises because the spectrograph is also used for many other programmes. Instrumental improvements and attention to detail has reduced this variability to negligible proportions. The disturbance of scintillation (2) is suppressed by averaging many sets of scan records. The water vapour problem (3) is eliminated by not observing in July and August. When these steps are taken the formal probable error for a 6-h run on C 5,380 is $\pm 0.15 \text{ K}$. The remaining fluctuation (4) is unavoidable and is due to the limb passage of faculae³ which display a 14-d periodicity with a peak amplitude of up to 4 K.

Figure 1 summarises the observational results through to the end of 1977. Our greatest confidence lies in the C 5,380 data. The facular component has been smoothed by forming 6-month averages excluding the summer season. The inverse relation between activity, as indicated by sunspot number, and deduced temperature, is clear. The change of mean values cannot arise from faculae alone, as the presence of the latter actually elevates the temperature at the time of increased solar activity. If we could remove the faculae the temperature decline would be even greater. Likewise, the contribution of sunspots is negligible because of their minuscule size and low emittance^{4,5}. Fe 5,379 qualitatively agrees with C 5,380, an interesting point because the sense of temperature change is opposite for the two lines.

Finally, a few words of caution. We measure a spectroscopic temperature change (observational fact) and then infer a luminosity reduction by assuming the Sun is a black body. But the Sun is really a grey body exhibiting limb darkening and other temperature inhomogeneities. We have no proof that the cooling is global. A very precise radiometry experiment is planned for the Solar Maximum Mission⁶ satellite in 1979. We may have to wait until then to 'calibrate' the C 5,380 global temperature sensitivity in terms of solar luminosity. Obviously this note is premature in that one would wish to have 11 yr of data before drawing conclusions, but the programme continues.

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Ignition by plasma jet

In order to improve the emission characteristics and efficiency of internal combustion engines, the trend is to leaner mixtures. Successful combustion at lower fuel: air ratios requires increased rates of flame propagation and, in spark-ignited engines, more effective and reliable ignition sources. Various avenues of research are being pursued towards these aims. One which shares the philosophy of radical injection with the present work is the stratified charge or torch-ignition concept (see ref. 1 for review). This is based on burning a rich mixture in a cavity small by comparison with the cylinder volume so that the free radicals generated therein are ejected into the main charge, there to increase the propagation rate in a leaner mixture. In the work described here the radicals are generated in the igniting plasma, rather than by using chemical energy, and the volume of the 'pre-chamber' of the stratified charge or torch ignition concept is shrunk to a very small space within the plug, where a very high temperature, resulting in high ejection velocity, is produced by means of a small amount of electrical energy.

Augmentation of combustion rates by radicals generated in discharge plasmas is not a new concept, for example, see ref. 2. The relative effectiveness of different plasmas for increasing combustion intensity has been studied in continuous flow, highly stirred systems, by Harrison and Weinberg³. In this work rapid

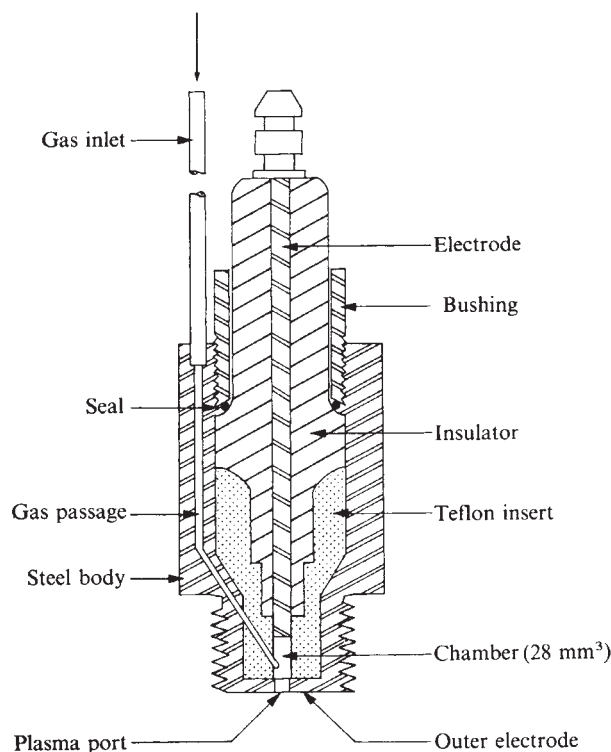


Fig. 1 Plasma jet ignition plug.

mixing was achieved by the application of a magnetic field which induced very fast rotation in the d.c. arc of the plasma jet. In these conditions, nitrogen atoms were found to be particularly effective, presumably because of their long life and high energy. Accordingly, nitrogen was also used in subsequent work,⁴ both for increasing burning rates and cleaning up pollutants from the combustion process—notably NO and soot.

The plasma jet used in the latter work was constructed from a sparking plug and no magnetic stirring was used. There is some indication from the later, as yet unpublished, phases of this work that hydrogen may be more effective than nitrogen in increasing burning rates. This does not contradict the findings of Harrison and F.J.W.³ detailed above, because in a system which is not highly mixed, the large diffusion coefficient of the hydrogen atom would be expected to confer an advantage which might overcompensate for those of the nitrogen atom. We stress these points because the choice of the most suitable plasma is crucial to our work and it is important to emphasise that the optimum choice may be determined by the extent of mixing in the system.

Relevant developments have also occurred in ignition research. Measurements of minimum ignition energies for plasmas of various compositions, generated by focusing laser beams on minute targets of different materials in flammable mixtures, have shown that results are independent of the make-up of the plasma only for pulses of very short duration⁵. Various designs of plugs which eject a small volume of plasma have been shown to be more effective ignition sources than sparks of comparable energy (see, for example refs 6-8), although none of these varied the plasma constitution.

In order to exercise the degree of freedom of controlling the radicals generated by varying the species in the cavity of the plug, we constructed the intermittent plasma-ejecting ignition plug illustrated in Fig. 1. The gas inlet was used to fill the cavity with the species under test immediately before triggering the discharge. This flow was metered and, although it was more than sufficient to fill the cavity, the overflow was calculated to have no appreciable effect on the composition of the main charge. This precaution was prompted by our objective of

comparing different radicals—in practical use a less transient flow through the plug may offer an additional means of improving ignition and subsequent flame propagation. The dimensions of the cylindrical cavity were 2.38 mm diameter and 6.29 mm depth, giving a volume of 28 mm³. The plasma ports were apertures of various diameters in interchangeable end-plates, attached to the electrode by three small screws.

The plasma was fired into a closed cylindrical stainless steel chamber, initially at atmospheric pressure, provided with 10 cm optical glass windows for high speed schlieren cine photography. These windows were 9 cm apart and formed two boundaries of the test space. The bomb, which was 9 cm in diameter so that the propagation could be followed all the way to the walls, was filled with a mixture containing 5% by volume of methane in air, which is outside the normal limit of flammability. The reason for using such a lean mixture was in anticipation that only a small proportion of the bomb's contents would burn, at least for normal ignition. The object was to obtain maximum discrimination between different igniting plasmas using, if necessary, much higher ignition energies than is current practice.

In the event, ignition by a conventional sparking plug never resulted in an appreciable proportion of the total volume being burned. Even when it dissipated 7.6 J the appearance was very similar to that illustrated in Fig. 2a. The energy dissipated in the discharge was determined from voltage and current wave forms measured on a calibrated oscilloscope. However, the effect of varying energy input was very small by comparison with the other plasma variables. Figure 2 illustrates energy inputs of 2.14 J and the results were almost indistinguishable from similar sets at 7.6 J and 0.15 J.

Various substances likely to produce radicals important in flame propagation were fed to the plasma plug, including hydrogen, nitrogen, water, fuel (methane) and fuel/air mixture (that is, no feed). Only liquid water was totally ineffective in producing ignition: the discharge seemed to produce atomisation and ejection of liquid droplets before the vapour could be heated in the plasma. With this exception, all plasmas were considerably more effective than normal spark ignition. However, experiments with the smallest apertures at the plasma port showed very little discrimination as between the different plasma species.

The main sequences were carried out with an aperture equal in size to the diameter of the cavity (0.24 cm) and with one half that size. With the latter, a very high-velocity jet was produced which projected to the opposite wall, the main ignition occurring on rebound. During the jet's emergence from the plug, flow shear at the interface is so intense that one can observe extinction of incipient flame fronts produced at that stage, even for a hydrogen plasma (Fig. 2b). This is not to rule out the use of the high-velocity jets for practical purposes—indeed the facility of varying the jet penetration, providing a source of ignition a controllable distance from the igniter, is clearly of considerable practical importance and it may well prove that a 'pepper-pot' arrangement with several such small apertures could provide a simple method of producing multi-point ignition from a single plug. The large area of the highly turbulent rebounding front certainly proved a very effective means of ignition and at energies of the order of 7.6 J always lead to complete burning of the charge. However, for our purposes it suffers from the disadvantage of not distinguishing between the different radical species, many of which are likely to have recombined by the time the jet rebounds from the opposite wall. If, as is confirmed below, such specific radicals can aid flame propagation, then this would seem to be the more efficient principle to use from the practical point of view also.

Figure 2c, d, e are taken with the larger aperture, of 0.24 cm, using the lean fuel/air mixture, hydrogen and methane feed respectively. The large differences in propagation rates are immediately obvious—note the widely different time intervals between the frames selected from our films. The simplest method, involving no extraneous supply to the plug, is the least

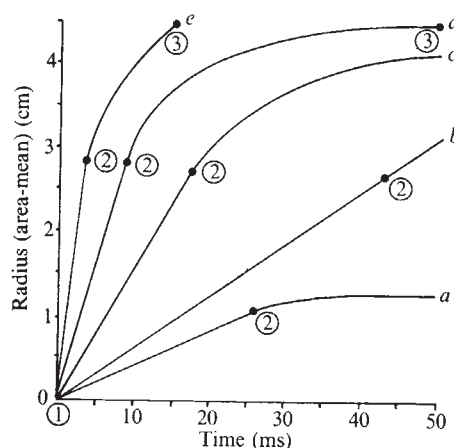
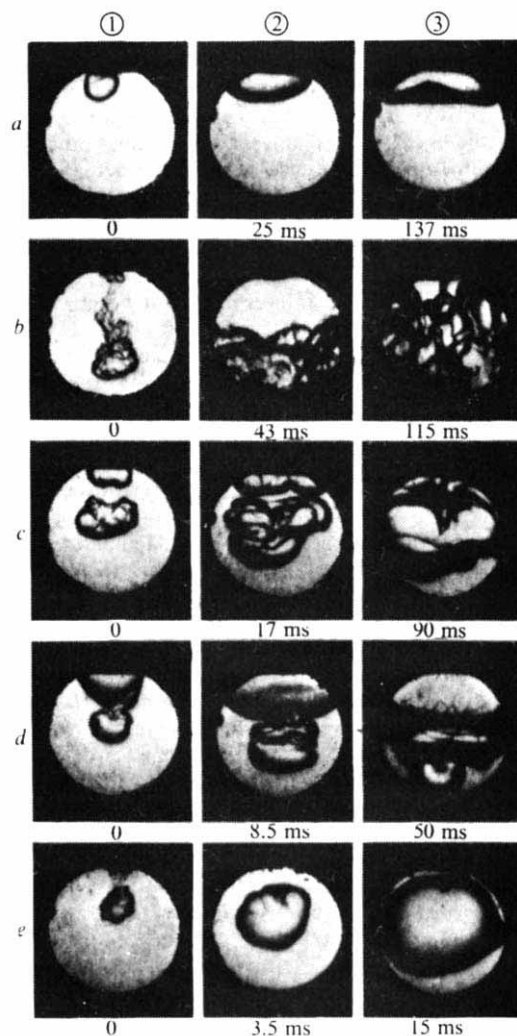


Fig. 2 Growth of flames following ignition by 2.14 J. a, Conventional spark; b, high velocity hydrogen plasma; c-e, lower penetration plasma jets of lean methane-air mixture (c), hydrogen (d), and methane (e).

effective (Fig. 2c). The addition of hydrogen gives rise to a very much larger rate of propagation (Fig. 2d), as would have been anticipated from the above discussion. In many ways the most interesting result, however, is the effect of pure fuel in the plug, which leads to very rapid symmetrical flame spread, resulting in complete combustion of the mixture in the minimum time (Fig. 2e). Calculation of the fundamental burning velocity from

the propagation rate of the expanding flame front yields a value approaching the burning velocity of a stoichiometric mixture, implying an approximately 50-fold increase in reaction rate in this normally nonflammable composition. This remarkable result is thought to be due to pyrolysis at the high temperatures within the plug producing more hydrogen atoms from fuel than from the same volume of hydrogen. The effectiveness of these three plasmas is, in fact, in the sequence of the amount of hydrogen atoms produced. However, the possibility that ultraviolet radiation from the plasma also plays a significant part cannot be ruled out at this stage.

From the practical point of view, this result has a particularly attractive feature: while it is not inconceivable that a vehicle might carry the small amount of hydrogen gas required to feed plasma plugs, it would clearly be much more convenient to use the fuel itself. With this point in mind, experiments were carried out with iso-octane (2, 2, 4-trimethylpentane) fed to the plug—with most promising results. More detailed results will be published elsewhere.

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Excess electrons in ice

CONSIDERABLE evidence has accumulated for a highly structured, ice-like nature of the aqueous medium in certain cellular systems, particularly in the vicinity of macromolecules such as DNA (refs 1–7). A consequence of this ordering of water molecules is that the dielectric relaxation time of the medium becomes considerably longer than the 10 ps or so found for normal water⁸. A correlation has been found in recent experiments between the time required for solvation of electrons in polar liquids^{9–13} and the dielectric relaxation time. On the basis of this a longer lifetime for unsolvated ('dry'¹⁴) electrons in biological systems, than the few picoseconds^{15,16} found in normal aqueous media, would be expected. An extended lifetime for dry electrons, particularly if coupled with a high electron mobility, would increase the probability of this species playing a role in cellular radiation damage, as has recently been suggested¹⁷, and also in intra- and inter-molecular charge transport processes in biological systems. To obtain an insight into the properties of electrons in a structured aqueous medium, we have studied the conductivity change resulting from nano-second pulse ionisation (irradiation) of ice. We outline here some conclusions we have reached concerning the yield and

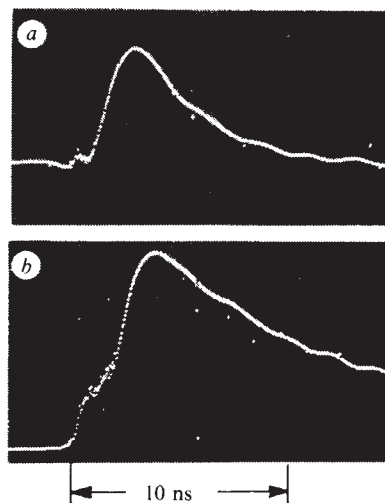


Fig. 1 Monitor traces of the decrease in microwave power reflected by an ice sample on irradiation with a 2.5 ns pulse of 3 MeV electrons (total dose per pulse approximately 60 rad). The maxima in the traces correspond to changes in the power level of ~ 3 and 5% at *a*, -42 and *b*, -61 °C respectively.

mobility of excess electrons as well as the processes of electron trapping and ion recombination in this medium.

Optically clear ice samples ($23 \times 10 \times 40$ mm) were ionised by irradiation with 2.5 ns duration pulses of 3 MeV electrons from a Van de Graaff accelerator. The transient conductivity change was monitored as the reduction in the known level of microwaves reflected by a short-circuited piece of X-band waveguide (0.2 mm thick, gold plated, stainless steel walls) in which the ice was contained. The microwave conductivity technique is fully described elsewhere¹⁸. The proportionality factor, relating the change in reflected power to the change in conductivity of the medium was derived from the known dielectric and geometric characteristics of the sample as has been described previously¹⁸. The ice sample was prepared from triply distilled water which was bubbled with helium and degassed on a grease free vacuum line before freezing in the waveguide. A detailed description of sample preparation will be given elsewhere.

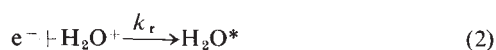
Figure 1 shows traces of the transient change in conductivity of an ice sample resulting from pulse irradiation. From the conductivity change observed and the known dosimetry, it is possible to evaluate the product of the yield of charge carriers formed per 100 eV absorbed, G , and the sum of the positive and negative ion mobilities, $[\mu(-) + \mu(+)] \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. This product is found to be $6.8 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1} (100 \text{ eV})^{-1}$ at -120 °C and increases somewhat to $8.3 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1} (100 \text{ eV})^{-1}$ at -40 °C. If a maximum value of $5(100 \text{ eV})^{-1}$ is taken for the yield of ion pairs, then a lower limit of $1.6 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ is obtained for $[\mu(-) + \mu(+)]$. This value is $\sim 1,000$ times larger than the value of $2 \times 10^{-3} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ which has been determined for the proton mobility in ice¹⁹. It is apparent, therefore, that at least one of the primary charge carriers (electron or hole) formed in irradiated ice is highly mobile.

That it is the electron which is the major charge carrier ($\mu(-) > 10\mu(+)$) has been demonstrated by preliminary results obtained using a coaxial, d.c. conductivity cell. The use of such a cell design to differentiate the relative contributions of negative and positive ions to the conductivity of a medium following pulse ionisation is described in refs 20,21. Using the same technique, from the effect of field strength on the decay of the current, the electron mobility could be estimated to lie within the range $20 \pm 10 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at -120 °C. The mobility of the dry electron in ice is therefore of the same order of magnitude as the Hall mobilities of electrons in alkali halide crystals²² and is somewhat higher than the values, of the order

of $4 \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$, found for the Hall mobility of electrons in glassy 10 M NaOH ice²³.

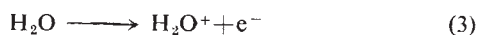
From the limits to the value of the mobility given above and the values of $G\mu$ obtained from the microwave measurements, the yield of free ion pairs can be estimated to lie within the range $0.23\text{--}0.70 (100 \text{ eV})^{-1}$ at 120°C . The yield of free ions in ice is hence considerably smaller than in water for which a value of $2.7 (100 \text{ eV})^{-1}$ is found. This has previously been suggested on the basis of a comparison of positron annihilation studies in the two media²⁴.

The traces in fig. 1 show that the conductivity signal, which is directly proportional to the concentration of dry electrons, has a limited and strongly temperature-dependent lifetime. This can be ascribed either to electron trapping (localisation) at defect sites in the lattice and/or to recombination of electrons with positive ions (initially assumed to be holes).



For temperatures of -80°C and lower, the first half life of electrons was found to depend almost inversely on the end-of-pulse electron concentration (dose in the pulse varied from 6 to 60 rad). Above -80°C , however, an increase in the end-of-pulse electron concentration was found to have a less marked effect on the electron half life, until at -40°C almost no effect was observed. Apparently for the doses used in the present experiments, ion recombination is the major electron removal process below -80°C but a pseudo-first-order trapping process becomes of increasing importance above this temperature.

A full kinetic analysis, based on reactions (1) and (2) and the formation process,



has been carried out and is found to give a good fit to the experimental data over the temperature range -40 to -120°C . It is found that if the value of k_r is taken by the Debye equation²⁵ for ion recombination to be $k_r = \mu/\epsilon_0\epsilon_r$, then the value of the relative dielectric constant, ϵ_r , required to fit the data is temperature independent and equal to 2.0 ± 0.1 . For a mobility of $20 \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$ and $\epsilon_r = 2.0$ the rate constant for ion recombination is $1.8 \times 10^{-5} \text{ cm}^3 \text{s}^{-1}$ ($1.1 \times 10^{16} \text{ M}^{-1} \text{s}^{-1}$). The value of ϵ_r found lies between the limiting high frequency (microwave) dielectric constant of 3.1 (ref. 26) and the optical value of 1.72 (ref. 27). A value of 2.0 is in fact found²⁸ for frequencies just above the translational band of ice (maximum 229 cm^{-1}).

The pseudo-first-order rate constant for trapping, $k_t N_t$ (where N_t is the concentration of traps), is found to increase from $4 \times 10^6 \text{ s}^{-1}$ to $4 \times 10^8 \text{ s}^{-1}$ in going from -80 to -40°C . This strong temperature dependence is considered to result from a high activation energy ($\sim 0.6 \text{ eV}$) for the formation of the defect site responsible for electron trapping. Doping of ice with 10^{-3} M NH_3 , results in a dramatic increase in the rate of electron trapping below -50°C . This suggests that Bjerrum D-defects play a role in the trapping process. However, this defect alone is insufficient to explain all of the results. In view of recent estimates of relatively high concentrations of vacancies in ice^{29,30}, we feel that a combination of a D-defect with a vacancy (a 'dressed' or 'vested' vacancy³¹) is the most likely trapping site for electrons. Both the available space and the excess positive charge associated with a dressed vacancy would favour electron localisation at such a site. Further deepening of the trap, leading to the 'solvated' state observed optically³²⁻³⁵, could subsequently occur by rotation of a single H_2O molecule followed by outward movement of the resulting L-defect.

In conclusion, an ice-like arrangement of water molecules provides a medium in which rapid transport of electrons can occur. The actual distances over which electrons can be transferred will depend on the extent of the structured medium, and the concentration of trapping sites. In this respect it is interesting that the DNA molecule which is considered to induce local structuring of water also apparently reduces the concentration of trapping sites in ice. Thus, an electron lifetime an order of magnitude longer than in pure ice is found in a frozen solution of the sodium salt of DNA ($5 \times 10^{-3} \text{ M}$ nucleotide concentration). Electron lifetimes of several nanoseconds, corresponding to total path lengths of several million angstroms, are therefore conceivable in the vicinity of such molecules. A fuller presentation of the results, and discussion of their relevance to other fields of research will be given elsewhere.

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Equatorial irregularity belt and its movement during a magnetic storm

GEOSTATIONARY satellites are able to monitor amplitude, phase, plane of polarisation (Faraday rotation) and group retardation of radio waves passing through the ionosphere with improved temporal resolution, although some of these parameters were earlier measured using orbiting satellites. An extensive experiment has been conducted on Faraday rotation measurements at a chain of stations covering a latitude region from the magnetic equator to 45°N dip (30°N geog.) in the Indian sub-continent using 140 MHz radio beacons received from ATS-6 satellite¹. We report here the first evidence of an irregularity belt and its movement during a magnetic storm.

The magnetic storm of 10 January 1976 was studied, and Fig. 1a shows the record of the horizontal component (H) at Kodaikanal (long 77.5°E , lat 10.0°N). The sudden onset of flux of about $+20\text{ nT}$ was seen at 1120 LT (75° EMT) and the range of H variation during the storm time was around 260 nT at Kodaikanal. After that night, severe Faraday rotation scintillations were observed at all stations except day rotation scintillations represent fluctuations in the day rotation scintillations represent fluctuations in the electron densities along the ray path of the radio waves. The longitudinal extent of the irregularity belt can be calculated from the time duration of the Faraday rotation scintillations by taking into account that the Earth rotates 15° of longitude in an hour. So if the time duration is

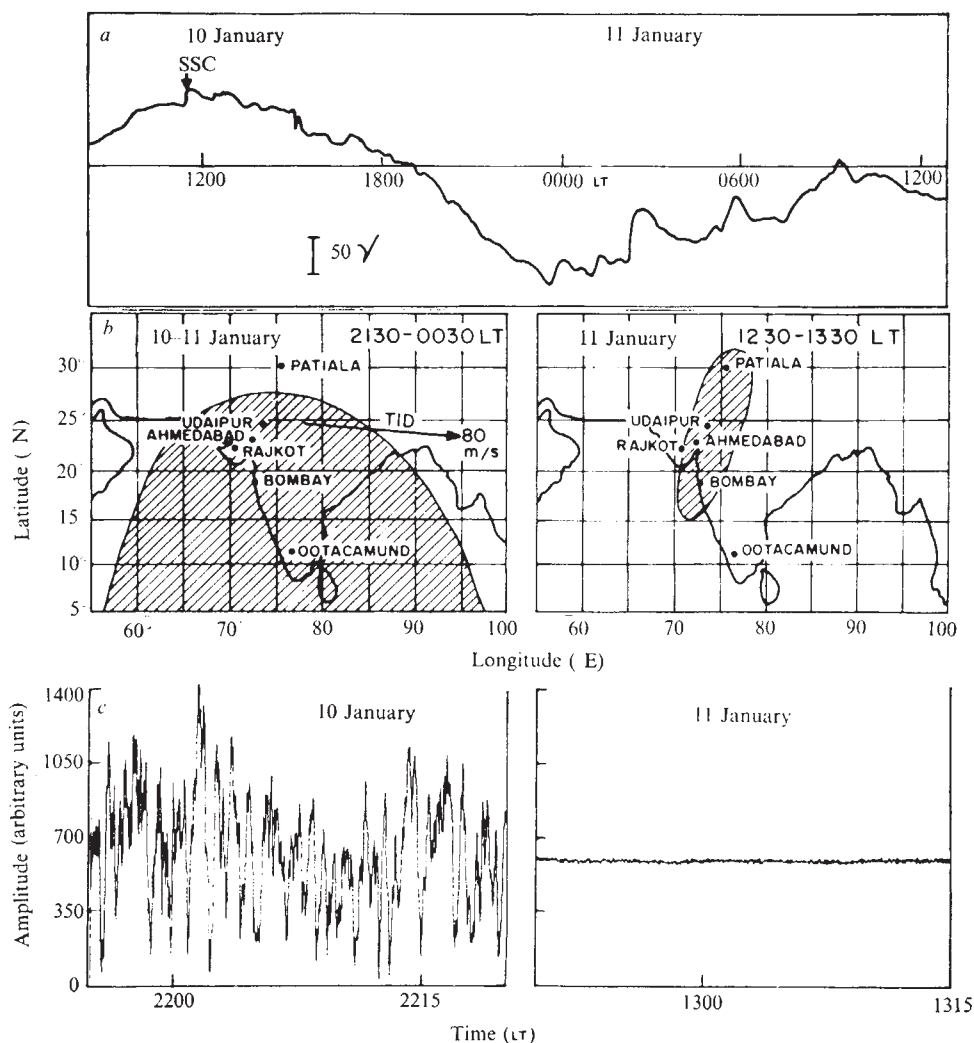
known the longitude extent can be calculated. This is shown in Fig. 1b. The shaded parts show the extent of irregularity belts.

During the main phase of the storm (2130–0030 LT) the irregularity belt extended from the magnetic equator to about 28°N (lat); whereas at normal spread-F nights the belt of strong scintillation zone centred on the magnetic equator is $8\text{--}10^\circ$ in the latitudinal direction². Travelling ionospheric disturbances (TIDs) were also observed with the large scale scintillations at this time. Using a triangulation method for TID data at Rajkot, Udaipur and Bombay, the speed and direction of TIDs were obtained. The motion is almost towards the east, with a magnitude of about 80 m s^{-1} , as shown in Fig. 1b. During the recovery phase of the magnetic storm (1230–1330 LT, 11 January 1976) another smaller irregularity belt was noticed extending from ~ 15 to 30°N (lat), with no scintillations near the magnetic equator. This suggests that the irregularity belt had shifted towards the north during the recovery phase.

The amplitude of 140-MHz radio beacons was also recorded at Ootacamund. These records were examined during the storm time. Fig. 1c shows the amplitude scintillation records in both the cases which agree with Faraday scintillations. The left-hand amplitude scintillation record shows that there can be severe disruptions in the trans-equatorial communication during the magnetic storm, as the signal fade during this time is about -22 db .

In high-latitude regions during magnetic storms the boundary of the irregularity belt moves to lower latitudes (or towards the equator) in a similar way to other auroral phenomena³. There the irregularity producing scintillations

Fig. 1 Effect of magnetic storm on: *a*, magnetic, H , field variation at Kodaikanal on 10 and 11 January 1976 showing storm with SSC at 1120 LT. *b*, Irregularity belts estimated from the Faraday scintillations observed at the chain of stations during the main phase (2130–0030 LT) and recovery phase (1230–1330 LT). *c*, Examples of the 140 MHz amplitude records at Ootacamund during the main, and recovery phase.



is probably due to particle precipitation which occurs to much lower latitudes during a magnetic storm. Generally, scintillations are frequent at Ootacamund but are not observed at other stations of the chain. We suggest, therefore, that the equatorial irregularity belt extended to $\sim 27^\circ\text{N}$ on 10 January 1976, during the magnetic storm. As far as fading rate is concerned there seems to be an agreement between the present and earlier observations at high latitudes. However, study is needed to determine the mechanics involved in the northwards movement of the boundary of the irregularity belt during a magnetic storm.

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Decrease in content of lead in Danish cereals

ENVIRONMENTAL pollution by lead, resulting in part from the use of petrol additives, is well established, and lead is known to be transferred through food chains¹. However, there has apparently been no previous long term study of the lead content of food. We have been able to make such a study by using samples of Danish food substances, which have been collected annually at the Health Physics Department of Risø National Laboratory. The samples are always dry ashed at 500°C for 24 h and then stored in polyethylene boxes with close fitting lids. Tests show that this causes no loss of lead or contamination by lead. So far we have examined three cereals (spring and winter wheat (*Triticum vulgare*) and barley (*Hordeum sativum*), and total diet², which is the average daily diet of an adult in Denmark. (The composition of total diet is given in ref. 2.) We report here that there has been an overall decline in the lead content of these food substances between 1962 and 1976.

The sampling programme, which was designed with special reference to an analysis of variance (anova) of the analytical results³, is explained in Fig. 1. Samples were

Table 1 Analysis of variance of $\log_e$ (μg lead per kg) in Danish cereals and total diet samples with respect to time of collection, location, and the interaction of the two factors

Sample	Source of variation	df	Mean squares	Significance
Barley	Year	14	0.76	99.9%
	Location	10	0.20	—
	Interaction	121	0.12	—
	Error	11	0.07	—
Spring wheat	Year	13	1.36	99%
	Location	10	0.81	—
	Interaction	52	0.45	99.9%
	Error	59	0.03	—
Winter wheat	Year	14	2.10	99.9%
	Location	10	1.10	99%
	Interaction	79	0.37	99.9%
	Error	82	0.04	—
Total diet	Year	8	0.60	99%
	Location	7	0.02	—
	Interaction	48	0.02	—
	Error	7	0.07	—

analysed by optical emission spectroscopy, the lines were recorded on photographic plates and their intensity was measured with a microdensitometer giving an accuracy of 10–20% for lead concentrations down to about 1 p.p.m. The results are summarised in Fig. 2, where each point represents the mean value found for samples from different locations.

The concentrations of lead found in cereals (~ 40 – $120 \mu\text{g}$ per kg fresh weight) are comparable with those reported for whole-grain products in New York⁴ (~ 50 – $130 \mu\text{g}$ per kg) and in the UK⁵ ($\sim 150 \mu\text{g}$ per kg). Lead in total diet varied from 15 to $8 \mu\text{g}$ per kg fresh weight. With an assumed daily per capita intake of 2.9 kg, including 1 kg of water, this corresponds to 43–23 μg per person per day. This is somewhat lower than the American⁴ and UK⁵ estimates of 70–80 and 165 μg per person per day, respectively. Our samples,



Fig. 1 Map of Denmark. Cereal samples were collected at 10 state experimental farms indicated by dots; one (marked by an asterisk) was closed during the sampling period and replaced by another (marked by two asterisks). For collection of total diet samples, the country was divided into eight zones corresponding to the counties as shown by heavy lines; in each zone, samples from six towns were pooled.

however, may not give a correct indication of the consumption of trace metals by the population, because the samples have not been prepared as food and have thus not been in contact with cooking utensils, which may well influence the content of trace metals in the food that is eaten.

Two-way (year, location) anovas on the analytical results (Table 1) confirm the tendency of the lead content to decrease during 1962–76, as shown in Fig. 2. Moreover, one-way anovas of values for food substances from individual farms show that lead concentrations were greatest during the early part of the study period in almost all cases. Concentrations have increased only in grain from one farm

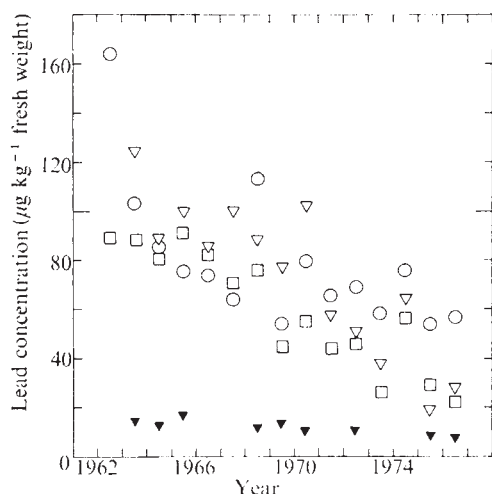


Fig. 2 Lead concentrations in three Danish Cereals and in total diet. The values refer to the fresh weight of samples that were later ashed, but otherwise untreated. Each point represents the average for the whole country. ○, Barley; □, winter wheat; ▽, spring wheat; ▼, total diet.

(Virumgaard) in an area north of Copenhagen where traffic is heavy: values were significantly larger (approximately 50% above average) than those for grain from other farms. This is part of the reason for the interaction between locations and years observed for the wheat varieties (that is, time variation showed a different pattern at the various locations). Unfortunately, grain has not been grown at Virumgaard since 1968, and so grain from another experimental farm (Fig. 1) was used instead. The exclusion of Virumgaard from the anova does not alter our conclusion.

During 1962–1976 petrol consumption in Denmark increased from 1 to 2.5×10^6 m³, and restrictions on the use of lead additives were not imposed until 1977. It is estimated that in 1975 the total lead content of petrol was 1,200 tons, of which about 900 tons were emitted into the atmosphere⁶; this is far more than can be assumed to originate from other sources. Nevertheless, we have observed a marked decrease in the lead content of some Danish food substances. A possible explanation is that in agricultural areas the direct uptake of lead from the air is generally small, and that lead in the edible parts of crops mainly originates from the soil⁷. In this respect it is interesting that the type of fertiliser used in Denmark has changed. Until the late 1960s, fertiliser was made of ammonium sulphate based on sulphuric acid produced by the lead chamber method; this resulted in lead contents of about 40 p.p.m. To a large extent this has been replaced by pure ammonia or ammonium sulphate produced by lead-free methods giving a lead content of only a few p.p.m. Moreover, more fertiliser is now generally used⁸, and this may influence the uptake of lead, which is not an essential element.

Although the larger than average concentrations of lead found in samples of grain from Virumgaard show that food contamination by car exhaust cannot be ignored, it is not

known how important it is, or whether it is influenced by other factors.

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Ultraviolet light detection by the homing pigeon

HUMANS cannot see the near ultraviolet (UV) wavelengths (320–400 nm) in sunlight. By analogy, it is often assumed that most vertebrates are also UV-blind¹. This generalisation has been called into question by recent experimental evidence showing that toads, newts, lizards, and hummingbirds are behaviourally responsive to UV light^{2–5}. We present here evidence suggesting that homing pigeons (*Columba livia*) are sensitive to UV wavelengths and capable of responding to the intensities of UV light that prevail in nature. Our results were obtained by a cardiac conditioning technique used previously to demonstrate homing pigeon sensitivity to barometric pressure changes, polarised light and infrasound^{6–10}.

Each bird was restrained by a leather harness and placed in a test chamber with a window through which the bird could view the light from a monochromator. A 10-s monochromatic light stimulus was presented at random times (10 per h) and served as a warning of an impending weak electric shock. The conditioned response of the bird was detected by an increase in heart rate occurring whenever the light was switched on.

The test chamber was an insulated 'picnic chest' (Thermos brand, 30 × 51 cm and 28 cm high) modified by installation of a 10-cm diameter pyrex optical window in one wall at pigeon eye level. Fresh air was pumped into the chamber through a small orifice to provide both ventilation and masking noise. A small lamp of about 1 L brightness illuminated the interior of the chamber. The chamber walls and each pigeon were surveyed with a geologist's UV lamp and shown to emit no visible fluorescence. The grating monochromator (Aminco 4-8460) produced a light beam adjustable to any wavelength between 300 and 750 nm. The beam had a constant band width of 18 nm at the half-power points. The lamp, a 600 W tungsten-halogen bulb (General Electric DYS), had an UV-transmitting quartz envelope. Lamp intensity was varied with a solid-state dimmer. Intensity was calibrated with the photocathode of a photomultiplier (RCA model 941) placed in the chamber at the site normally occupied by the bird's eye during testing. For wavelengths encompassing the UV range (300–411 nm), a bandpass filter (Zeiss U5) was inserted between the lamp and the monochromator to suppress harmonics and spurious reflections. The pigeon normally viewed the light source with the lateral field of the left eye. It could view the light beam with both eyes, but rarely did so because the optical path length was approximately 1 m, and pigeons prefer to view objects at this distance with the lateral field of one eye only¹¹.

A conditioned cardiac response to the light could usually be observed after no more than three trials. Responses occurred

when the lamp was turned on, but before actual delivery of the electric shock (1 MA, 60 Hz a.c. for 0.35 s). A positive cardiac response was scored if there was an increase in heart rate of at least 12 beats per min during light stimulation. The baseline heart rate was the highest rate recorded in the 5 s preceding the light. Baseline heart rates were typically about 140 beats per min; heart rates induced by light were in the range of 170–200 beats per min. Heart rate was measured with a tachograph (Grass Instrument model 7P4) connected to two EKG surface electrodes, and recorded with a chart recorder (Sanborn 350).

Intensity thresholds were determined by finding the lamp intensity at which a pigeon failed to respond 50% of the time. At light intensities higher and lower than threshold, the incidence of response was above and below 50%, respectively. Light intensity was varied over a 5-decade range.

Six pigeons were tested in the apparatus, and each was found to be highly sensitive to UV wavelengths in the range of 325–360 nm. Environmental sunlight intensity at UV wavelengths is of the order of 10^{-2} W cm $^{-2}$ nm $^{-1}$. Threshold intensities at 325 nm for all six pigeons were found to be lower than this level by several orders of magnitude. The least sensitive pigeon (no. 3064) had a threshold of 10^{-7} W cm $^{-2}$ nm $^{-1}$, while four others had thresholds of 10^{-9} W cm $^{-2}$ nm $^{-1}$. The sixth pigeon (no. 8269) was more sensitive at 325 nm than our photomultiplier tube (10^{-10} W cm $^{-2}$ nm $^{-1}$).

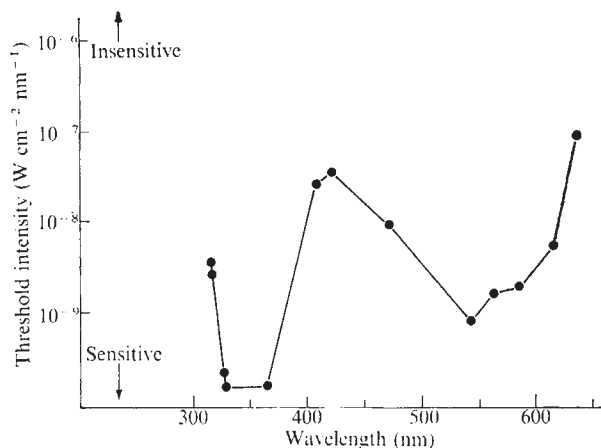


Fig. 1 Spectral sensitivity of pigeon 3075. Each point is a behaviourally determined threshold (50%). Details in text.

Control trials with four pigeons (including no. 8269) confirmed that the birds were responding to UV light and to no other cues. During the UV tests, a high-pass optical filter was occasionally inserted into the light pathway. This filter (Blak-Ray UVC-303) had excellent transmission for visible light but was opaque to UV light. It is clear from the data (Table 1) that with the filter inserted the UV-responses of the birds were essentially abolished.

To test for spectral differences in sensitivity, pigeon 3075 was conditioned at 13 different wavelengths spanning both the visible and UV region of the spectrum. The results (Fig. 1) showed the region of maximal UV sensitivity (325–360 nm) to be sharply defined relative to the conventional peak of sensitivity in the blue-green region (500–600 nm). The other birds were studied in less detail, but all proved to have sensitivity maxima in both the UV and blue-green regions; the UV maximum showed some individual variability in spectral span and location.

Ultraviolet sensitivity in homing pigeons had not been demonstrated directly, although Wright¹² noted that UV light affected the colour discrimination ability of his birds. Delius (personal communication) has independently found that the spectral sensitivity of homing pigeons includes a sensitivity peak in the UV region.

Nothing definite can be said about the mechanism of UV

Table 1 Cardiac responses of UV-conditioned pigeons to UV light-stimuli, blocked or unblocked by UV-filter

Bird no.	Without filter (responses/trials)	With filter (responses/trials)
3075	40/40	2/33
6982	14/16	0/10
8269	23/25	3/25
7778	20/21	0/24

Pooled data are shown for the two wavelengths (325 and 387 nm) to which the birds were conditioned. The light source was set to maximum intensity and the filter occasionally inserted into the light path. Filter (Blak-Ray UVC-303) had 80% or better transmission to wavelengths longer than 420 nm, but less than 0.1% transmission below 405 nm.

detection in homing pigeons. Assuming the eyes to be the receptors involved, UV light could be detected either directly by photopigment(s) in the retina, or indirectly by induced fluorescence. In humans, the eye lens fluoresces when exposed to intense UV light. This produces a visible illumination in the interior of the eye known as the 'blue haze'. However, no image is formed, nor can the light source which generates the internal light be seen¹³. To form images, UV light must reach the retina. Ultraviolet-absorbing pigments in the human lens exclude short wavelengths from the retina. If the lens is removed, UV light can reach and excite the retina. Individuals surgically deprived of their lens (as through a cataract operation) can read an optometrist's chart with 365 nm light alone¹⁴. Since our pigeons detected UV light at intensities lower than those required to induce 'blue haze', we are inclined to rule out visible fluorescence as the mechanism involved. The lenses of pigeons and some other birds are relatively transparent to UV light, as are their corneas and ocular media¹⁶. It is conceivable, therefore, that UV wavelengths are sensed directly by their retinas, although ultimate proof is contingent upon demonstration of UV-image detection. Clear lenses are also said to occur in toads (*Bufo bufo*)³ but not in frogs (*Rana pipiens*)¹⁴, and in some, but not all, fish¹⁵. Ultraviolet light might therefore reach the retina of more vertebrates than has been suspected previously.

We do not know in what adaptive contexts UV detection might be used by pigeons. Invertebrates such as insects, in which visual sensitivity to UV light is well known, use UV cues for location of the sun behind thin clouds, for orientation to polarised light patterns in the sky, and for detection of UV patterns on flowers and mates¹⁷. Whether the world holds comparable meaning in the UV for some vertebrates remains to be seen.

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Facultative sex ratios and population dynamics

THE theory of R. A. Fisher¹ for the effect of natural selection on the sex ratio predicts the population to be in evolutionary equilibrium when half of the parental reproductive resources are devoted to sons, and half to daughters. Provided that sons and daughters are of equal cost, this implies a sex ratio of 1/2 males at or near conception, and the result is independent of differential male and female survival after the period of parental care²⁻⁵. Most models demonstrating these results assume discrete generations, but some show that they also follow with overlapping generations⁶⁻⁸. These latter models typically assume the population to be in stable age distribution. Here, we relax that assumption, and show that selection can favour genes which result in the temporary overproduction of one or the other sex, under certain general conditions. The conditions are; overlap in generations; different temporal changes in life history expectations for the two sexes; parental ability to vary the sex ratio in response to the life history changes. The results are of interest because stable age distributions are probably uncommon in nature, with fluctuations in survival more the norm.

To investigate sex ratio selection under these new conditions, two models were developed, a cyclical model and a perturbation model. In the cyclical model, life history expectations for males and females vary cyclically over time, perhaps because of seasonality. Consider the following simple example. Let individuals be born in the spring and the autumn. Males and females born in the spring survive until the autumn. They then reproduce and die. Of those born in the autumn, the females survive to reproduce in the spring (and then die), while the males survive to reproduce in the spring and some survive to reproduce again the following autumn. Life history expectations differ for individuals born in the spring versus the autumn, and some overlap exists in generations (Fig. 1). This example is extreme in that only one sex (σ) reproduces twice (so that spring-born males face reproductive competition from males born the previous autumn, while spring-born females face no such competition).

If r_t and r_s are the sex ratios (proportion males) born in the autumn and spring respectively, it is easy to show (using computer simulation or analytical population genetics) that the sex ratios favoured by natural selection are such that $r_t > 1/2$ while $r_s < 1/2$. As the amount of competition faced by spring-born males for mates in the autumn increases due to the presence of autumn-born males of the previous year, the deviations in sex ratios increase from 1/2. Figure 2 shows this effect of increasing overlap upon the equilibrium sex ratios among spring-born and autumn-born, as derived from an analytical population genetics treatment of the life history outlined above.

A more extensive treatment of our model, with consideration of other seasonal life histories (such as a model where both males and females have overlap in generations) will be published elsewhere. However, the model just presented captures the basic points. Selection can favour seasonal shifts in sex ratio if males and females experience different life histories which vary seasonally, and, because of overlapping generations, those born at different times compete with each other for relative reproductive success. This creates a situation where a parent may 'derive' more fitness by shifting the sex ratio towards offspring of the sex with improved reproductive success relative to the other sex. We note here that if facultative sex ratios are not possible (so that $r_s = r_t$), our analysis shows that the equilibrium sex ratio returns again to 1/2 males.

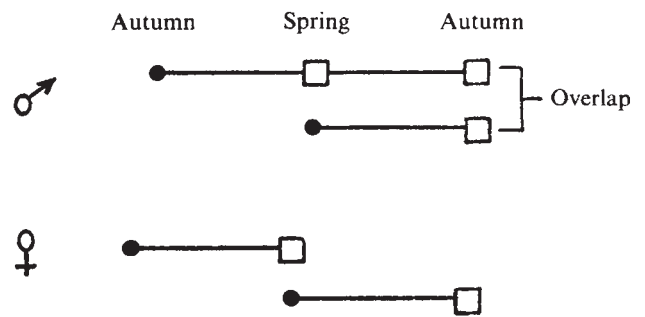


Fig. 1 Diagrammatic representation of the hypothetical set of life histories in a seasonal environment. ●, Born; □, mating period.

The model just presented assumed the life history characteristics to vary seasonally. We now consider a perturbation model. Assume that a population with overlapping generations has a stable age distribution, and suppose that the population is occasionally perturbed by a period of exceptional mortality or survival, followed by a return to 'normal conditions.' These sorts of perturbations may be common in nature; they can be caused by dramatic weather changes, such as cold or heat spells and floods. In species subject to strong sexual selection, one sex can be affected considerably more than the other by such occurrences, due to behavioural and/or physiological differences. Males are known to be more vulnerable to environmental stress than females in many species^{9,10}. The perturbations must be dramatic and fairly frequent if they are to select for facultative sex ratios. The question follows: does selection favour genes which allow an individual (mother or father) to respond to the change from stable age distribution conditions by overproducing the sex which suffered the greater mortality (in terms of deviation from normal survival)? We investigated this question using computer simulation.

Let us suppose, for example, that the perturbation caused young males to suffer twice their normal rate of mortality between birth and age one. A gene which biases sex ratio towards males in the next generation would be favoured because of the reduced competition faced by those males relative to their own expected mortality. On the other hand, if females suffered twice their normal rate of mortality, selection would favour biasing sex ratio towards females in the next generation. Again, if the facultative response is not possible (a single sex ratio is used all the time), our computer results indicate that 1/2 is the equilibrium value, and that the violation of stable age distribution does not itself alter the value from that predicted under Fisher's theory.

We have discussed both these models in terms of varying ability to survive; however, the results should also hold for other fitness components which vary over time (such as competition for territories; do early spring-born males procure the best territories?). The basic difference between these two models is that the life history changes are predictable in the cyclical model and unpredictable in the perturbation model. Therefore, in the latter case the sex ratio response can only occur after the life history change. Both models predict that selection will favour a shift in offspring sex ratio towards the sex with improved reproductive success relative to the other sex.

Our theory requires that animals or plants are able to alter the sex ratio among their offspring, and that they can do it at the appropriate time (so that there must exist appropriate cues). We indicate here some case histories of particular interest.

Haplodiploidy (fertilised eggs produce females, unfertilised eggs males) occurs among Arthropods and gives

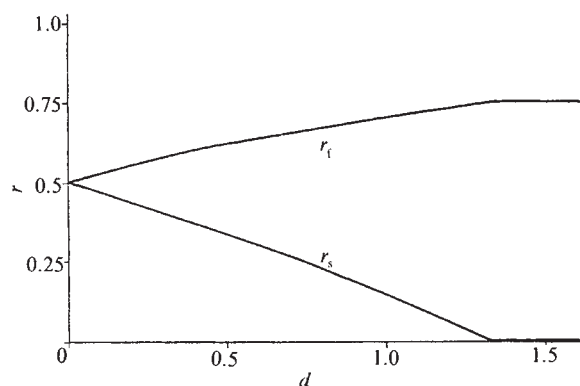


Fig. 2 The equilibrium solutions for autumn sex ratio (r_t) and spring sex ratio (r_s) as a function of d , a measure of the competition for mates faced by spring-born males in the autumn due to the presence of autumn-born males. Specifically, if N_t is the number born in the autumn, N_s the number born in the spring, S_t the survival of autumn-born males to the following autumn, S_s the survival of spring-born males to autumn and C the competitive ability of autumn-born males relative to spring-born males in gaining mates during the autumn, then $d = C(N_t S_t / N_s S_s)$.

As d increases, the reproductive success of spring-born males decreases.

the female much control over the sex ratio of the brood^{11,12}. There is evidence for temporal shifts of sex ratio in some parasitic Hymenoptera^{12,13}. Photoperiod (a seasonal cue) affects progeny sex ratio in the parasitoid wasp *Pteromalus puparum*. Under LD10:14, two to three times as many female progeny are produced than at LD14:10, although fertility is unaffected¹⁴. In another parasitic wasp, *Campoletes perdisticus*, the greatest percentage of females is produced under LD12:12 (ref. 15). Among mites of the genus *Macrocheles*, offspring sex ratio varies inversely with the adult sex ratio^{16,17}. Such a response is predicted under the perturbation model.

Life history expectations vary during the population cycle of multi-voltine rodents and these changes are different for males and females¹⁸⁻²². Juvenile sex ratios have been found to change with the population cycle in several species²¹⁻²³, however, this is difficult to interpret because of potential bias. The effect could be due to changes in behaviour, mortality after weaning, or rates of maturation. In one case, litter sex ratios of field populations of the bank vole (*Clethrionomys glareolus*) were examined by bringing pregnant females into the laboratory. The sex ratio of resulting litters was found to vary significantly with the population cycle²⁴.

There are several phenomena consistent with the prediction of the perturbation model that a scarcity of one sex leads to its overproduction in the next generation. It has been demonstrated experimentally in the guppy (*Lebistes reticulatus*) that offspring sex ratio varies inversely with adult sex ratio²⁵. In the plants *Silene dioica*²⁶ and *Rumex acetosa*²⁷, the sex ratio of seedlings varies inversely with density of pollen on the stigma. The physiological mechanism is speculated to be slow pollen tube growth of Y-bearing pollen. When pollen densities are low (a male perturbation?) the Y-bearing pollen has an improved chance of fertilising ova. In a small population, the effect of random sampling on the adult sex ratio would have the same influence as a perturbation, therefore favouring a facultative response.

Another line of evidence for the perturbation model is the effect of delayed fertilisation on sex ratio. In many organisms, a delay in mating or fertilisation causes a dramatic increase in the proportion of males in the progeny. This is exactly to be expected if the mating delay reflects an abnormal scarcity of males. Hertwig²⁸ showed in the anuran *Rana exculenta* that the sex ratio of late fertilisation

eggs gave a much greater proportion of males. Kuschakewitch²⁹ demonstrated that the result was not due to differential mortality (according to Huxley³⁰ and James³¹). Delayed fertilisation leads to a predominance of males in the trout (*Salmo iridens*)³², *Drosophila melanogaster*³³, several species of mealy bugs (*Pseudococcus*)³⁴, a butterfly (*Talaeoprois tubulosa*)³⁴, and three species of copepod (*Tisbe*)³⁵.

In the mealy bug, mating 0, 6, 8 and 10 weeks after emergence resulted in 102, 181, 327 and 991 males per 100 females, respectively. Sex ratio was ascertained at the prepupal stage; however, James³¹ showed that the changes in sex ratio were not due to differential mortality between conception and the prepupal stage, and therefore must be due to prezygotic mechanisms.

Certainly, more detailed life history information is necessary to test the models conclusively; however, the examples mentioned are suggestive. Experiments are now being carried out in an attempt to select for facultative sex ratios in *Drosophila melanogaster*.

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Precocene II causes atrophy of corpora allata in *Locusta migratoria*

BOWERS *et al.*¹ found that chromene derivatives extracted from the plant *Ageratum houstonianum* have 'anti-juvenile hormone' effects. They named the most active substance (6,7-dimethoxy-2,2-dimethylchromene) precocene II and demonstrated its 'anti-gonadotropic' activity in several insect orders, but obtained morphogenetic effects (precocious-prothetelic adults after administration to young instars) only in Heteroptera. Pratt and Bowers² have shown that precocene II inhibits juvenile hormone (JH) biosynthesis by cockroach corpora allata (CA) *in vitro*. Preliminary experiments³ have indicated that it has a morphogenetic anti-juvenile effect in the migratory

locust, *Locusta migratoria migratorioides*. We report here that precocene II, as far as investigated, induces all of the anti-juvenile effects in this insect and, at least in adequate doses, causes atrophy of the CA.

Some experiments were performed in Jerusalem and some in Wageningen. Crowded locusts were kept in a regime of 12 h light and 12 h darkness at 27 °C during the scotophase and 38 °C (Jerusalem) or 37 °C (Wageningen) during the photophase. Relative humidity was rather high, but uncontrolled in Jerusalem; it was maintained at 30–40% in Wageningen.

In Jerusalem we applied precocene II topically (1:10 w/v solution in acetone) or injected it in oil (1:10 w/v saturated solution in a 1:1 mixture of paraffin oil:olive oil) to third or early fourth instar nymphs. Results obtained after injections are not detailed here. In general, injection slightly improved the effect, but resulted in high mortality. Because controls injected with oil mixture alone also showed high mortality, we presumably 'overloaded' the nymphs with oil due to the limited solubility of precocene II in the mixture.

Precocene II applied to third instar nymphs had one of the following effects (Table 1). (1) It greatly delayed the moult and produced precocious-prothetetic fourth instar nymph-adult intermediates, termed fourth instar adultiforms, with advanced adult characteristics; such intermediates were permanent, viable and survived for at least 2 months. (2) It delayed the moult and produced superficially almost normal fourth instar nymphs or fourth instar adultiforms with moderate adult characteristics; these locusts, however, died at the moult from the fourth to the fifth instar. Death occurred within the exuvia, which either did not rupture, or did rupture but could not be shed by the insects due to the thickness of the (old) cuticle and to the already developed adult apodemes, apophyses and sulci, especially in the thorax. (3) In the cases of less intense effect, there was no delay, or only a slight delay, in the moult and superficially normal fourth instar nymphs were obtained. These nymphs, however, exhibited a subsequent (delayed) moult to permanent, precocious-prothetetic fifth instar adultiforms which were viable and survived for at least 2 months.

The CA exert complete control over the development of the yellow colour (for literature see ref. 4) characteristic of sexually mature crowded males of *L. migratoria* and over vitellogenetic oocyte growth⁵. We therefore investigated the effects of exogenous JH on these processes in locusts treated with precocene II.

Fifth instar permanent adultiforms were injected with JHIII (Fluka, Switzerland). Single doses of 15 µg hormone per 7.5 µl olive oil were injected every second day until the desired cumulative dose (45 µg JHIII: three repeated injections per male, or 75 µg JHIII: five repeated injections per female) was attained. Controls received either olive oil alone, or no injections.

Of six males receiving JHIII, all showed intense yellow colour 10–14 d after the first injection and maintained it for 4–5 weeks. Then the yellow colour started to fade. None of the five controls showed any yellowing for up to 2 months (end of the experiment) after moulting to adultiforms. The effect of JH on yellowing of the male adultiforms agreed well with the effect described by Staal⁶ after implantation of CA into adultiforms obtained by surgical removal of the CA.

Out of four fifth instar adultiform females receiving JHIII one laid a miniature egg pod (seven eggs) on the 9th day after first injection. During the next 3 d numerous holes in the sand indicated intense digging, but no further pods were found. On the 12th day after the first injection the females were killed and dissected. Three of them had respectively 17, 13 and 4 chorionated eggs in the oviducts and 2.50 ± 0.24 , 3.33 ± 0.32 and 3.28 ± 0.27 mm (average $\pm$ s.d.) long late vitellogenetic proximal oocytes in the ovarioles. The last female revealed no chorionated eggs and only four late vitellogenetic proximal oocytes (length, 4.56 ± 0.27 mm) in the ovarioles. Many proximal oocytes in these females were found in various stages of resorption. Controls (three fifth instar adultiforms) had small previtellogenetic proximal oocytes (0.416 ± 0.036 , 0.624 ± 0.036 and 0.800 ± 0.057 mm). The cumulative dose of JHIII injected into the adultiform females constituted a slight overdose, but still corresponded to doses inducing completion of oocyte development in surgically allatectomised adult female *Locusta*⁷. The timing of the process was also similar, but considerably fewer oocytes attained full development in these adultiforms than in allatectomised and JH-injected adult females (in the latter the number is in any case fewer than in normal females⁷). The synchronous presence of chorionated eggs and late vitellogenetic oocytes, as well as the fact that only one egg pod was laid in spite of the intense digging, may indicate that JH-injected adultiforms had difficulty in laying their eggs.

In the experiments at Wageningen where precocene II (same concentration in acetone as in Jerusalem) was applied topically to fourth or early fifth instar nymphs, the duration of the fourth instar in normal or acetone-treated controls was 5.75–6 d. Mortality was dependent on dose, and no less on age of application; from the 3rd day of the fourth instar, however, even 400 µg per nymph was not markedly toxic (Table 2).

Of 51 surviving locusts treated on the 1st day of the fourth instar with 100, 200, 300 or 400 µg precocene II (Table 2), 50 moulted to permanent adultiforms with advanced adult characteristics (3 or 4 in Fig. 1), and one to a permanent adultiform with moderate adult characteristics (2 in Fig. 1). Of 39 surviving locusts treated on the 2nd day of the same instar, 25 moulted to advanced permanent adultiforms, 10 to permanent adultiforms with slight or moderate adult characteristics (1 and 2, Fig. 1) and four to more or less normal fifth

Table 1 Mortality, morphogenetic and time effects caused by precocene II topically applied to early 3rd and 4th instar nymphs of *Locusta* in Jerusalem

Instar and age (h) at application		3rd 19–45		3rd 17–47		3rd 22–43		4th 21–48
Dose of precocene II per nymph (µg)		0*		100		200		400
No. treated		20		26		20		20
No. dead†		2		3		5		3
Instar and state reached		Normal adults	4th Instar adultiforms	Death at moult to 5th instar‡	5th Instar adultiforms	4th Instar adultiforms	Death at moult to 5th instar‡	5th Instar adultiforms
No. reaching this instar and state		18	8	7	8	5	10	17
Average duration of nymphal instars (h)	3rd	115	203	147	133	210	151	Not recorded
		(94–141)	(156–236)	(128–178)	(118–142)	(171–237)	(121–212)	
	4th	132	Permanent	243	205	Permanent	327	
(range)		(108–206)		(195–369)	(171–244)		(212–460)	186
	5th	222	—	—	Permanent	—	—	Permanent
		(168–383)						

* Controls received 2 µl of acetone.

† Mortality mostly occurred within 3 d of treatment, but all kinds of mortality are included, except that at the moult to the 5th instar.

‡ All died within the exuvia.

Table 2 Mortality and morphogenetic effects caused by precocene II applied topically to 4th and 5th instar nymphs of *Locusta* in Wageningen

Instar and day of application†	Dose of precocene II per nymph; (μ g)	Dead‡	Number of locusts§		Morphogenetically normal adults
			Permanent adultiforms	Dead at moult to 6th (adult) instar	
4th 1	0*	5	0	2	23
4th 5	0*	3	0	1	24
4th 1	100	5	25	No moult to 6th	0
4th 1	200	17	13	No moult to 6th	0
4th 1	300	26	4	No moult to 6th	0
4th 1	400	21	9	No moult to 6th	0
4th 2	200	6	20	2¶	0
4th 2	400	11	15	2¶	0
4th 3	200	1	1	12	14
4th 3	400	4	3	10	11
4th 4	200	1	0	0	29
4th 4	400	8	0	5	17
4th 5	200	1	0	1	26
4th 5	400	6	0	1	21
5th 1	400	1	0	3	26

* Controls received acetone alone.

† The period of 0–24 h after the moult is defined as day 1.

‡ Mortality mostly occurred within 4 d of treatment, but all kinds of mortality are included except that at the moult to the 6th (adult) instar.

§ Each experimental group comprised 28 or 30 locusts (50% males and 50% females).

¶ All locusts died within the exuvia.

|| Most locusts died either within the exuvia or due to inability to shed off the exuvia smoothly.

instar nymphs, which died within the exuvia at the next moult. Only four permanent adultiforms (2 or 3 in Fig. 1) were obtained with locusts treated on the 3rd day; two adultiforms (1 in Fig. 1) were not permanent and died at the next moult together with 20 more or less normal fifth instar nymphs. In most instances

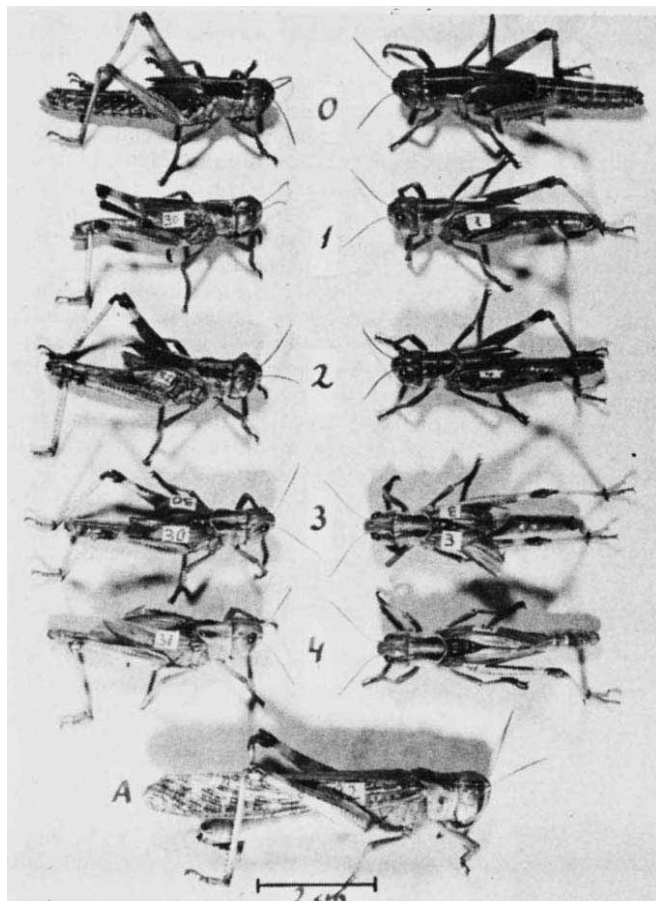


Fig. 1 Fifth instar permanent adultiforms of *L.m. migratorioides* obtained by treatment with precocene II in the early 4th instar compared with acetone-treated controls (Wageningen). 0, Normal 5th instar controls; 1, adultiforms with slight adult characteristics; 2, adultiforms with moderate adult characteristics; 3 and 4, adultiforms with advanced adult characteristics. The forewings are dorsal to the hind wings in (3), but in an outer position (as in normal adults) in (4). A, normal adult control.

this death occurred either within the exuvia, or due to an inadequate moult. The remaining 25 locusts moulted to morphologically normal adults as did all the surviving locusts treated on the 4th or 5th day of the fourth instar or the 1st day of the fifth instar (Table 2). The timing of all these moults again indicated that more marked morphogenetic effects are correlated with more delayed moult(s).

Within 2–3 weeks of fledging, all acetone-treated control males turned yellow and all control females showed yellowing on the hind wings. On the 11th–12th day after fledging controls started to oviposit at 2.0 pods per female per week for one group and 1.9 for the other.

Of 144 precocene II-treated morphogenetically normal adults (Table 2, last column), 126 survived to the 4th week of adult life. None of the males showed yellowing, and the hind wings of only three females (out of 68) became yellow. Five groups of locusts laid no eggs. The 6th group (14 females treated with 200 μ g precocene II on the 4th day of the fourth instar) included two of the females with yellow hind wings and laid altogether six pods during 2 weeks (starting from the first laying). The last group (11 females, 400 μ g precocene II on the 5th day of the fourth instar) included one female with yellow hind wings and laid four pods during a similar period. On the assumption that only the females with yellow hind wings laid, but at a normal rate, these numbers correspond well with the oviposition rate of the controls.

During the 4th week 49 females were killed and their ovaria were dissected. In 43 of them there were only previtellogenic proximal oocytes without yolk. In three females 1.3–1.6-mm long proximal oocytes with yolk, but no 'yellow follicles' (which indicate previous laying*) were found. The three females which showed yellowing of the hind wings also had proximal oocytes in various (3.6–6.5 mm) stages of late vitellogenesis and 'yellow follicles', confirming that the egg pods obtained had been laid exclusively by these females.

Nineteen females (400 μ g precocene II on the 4th day of the fourth instar or on the 1st day of the fifth instar) were kept for 67–71 d after fledging. No egg pods were found and there was no yellowing of the hind wings. Some of the similarly treated males (some others were dissected for examination of the CA, see below) kept with these females did not show yellowing either.

During the fourth week and onwards after fledging the retro-cerebral complexes of 16 precocene II-treated morphogenetically normal adults and of 10 adultiforms were dissected out under physiological solution. In all cases the CA had atrophied drastically (Fig. 2). It was impossible to see the glands *in situ*

under the dissecting microscope; as well as being extremely small (much smaller than in normal fifth instar nymphs), they were covered by fat.

These results demonstrate the morphogenetic anti-juvenile activity of precocene II and its anti-gonadotropic effect in an orthopteroid insect. The substance affects the CA and, at least in appropriate doses, causes them to atrophy, apparently irreversibly, in almost all locusts treated. Otherwise precocene II does not seem to have easily discernible effects, although it is toxic in larger doses (depending on the time of application). Once chemically allatectomised by precocene II, crowded adultiforms of the migratory locust react to injected JH as do surgically allatectomised animals; males become yellow and vitellogenesis is completed in the females. Thus, precocene II does not seem to prevent the target tissues responding to JH, as known already for Heteroptera¹.

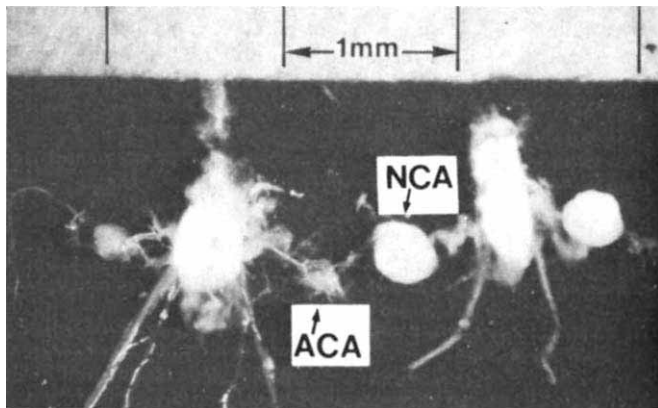


Fig. 2 Left, retrocerebral complex of a morphogenetically normal adult *L.m. migratorioides* male, treated with precocene II on the 1st day of the 5th instar and dissected 37 d after fledging. ACA, atrophied CA; the left one (not marked) with some adherent fat. Right, retrocerebral complex of an acetone-treated control male of the same age. NCA, normal CA. The retrocerebral complexes were dissected under physiological solution, fixed in 10% formalin for 16 h, transferred to 70% alcohol and then photographed. Experiments were carried out in Wageningen.

It remains to be seen whether the atrophy of the CA is a primary effect of precocene II in the sense that the substance selectively and actively kills the gland cells, or whether it is secondary and the atrophy results from lack of functioning due to a possible permanent inactivation of one or more components of the enzyme system(s) responsible for JH biosynthesis. Pratt and Bowers' findings² can be reconciled with either possibility.

It is interesting that precocene II induces atrophy of the CA, even when given to nymphs too late to obtain morphogenetic effects. This may be important for the practical application of precocene II or of substances with similar biological action—the 'fourth generation of insecticides' Bowers *et al.*¹

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The origin of lymphoid stem cells studied in chick yolk sac-embryo chimaeras

THE development of lymphoid organs in the chick embryo is dependent on colonisation by extrinsic stem cells^{1–6}, some of which are present in the yolk sac blood islands, as Moore and Owen demonstrated by transplanting 7-d yolk sac cells into 14-d-old irradiated chicken embryos². These authors demonstrated population of lymphoid organs by donor-derived cells, and suggested that all haemopoietic stem cells are formed in the early yolk sac³. This interpretation has been challenged^{7–11}, particularly on the basis of results obtained in quail-chick chimaeric germs⁷. We present here further evidence, based on the use of chick-chick yolk sac-embryo chimaeras, that lymphoid stem cells in the chicken do not originate primarily in the yolk sac.

In the developing avian blastoderm, two clearly defined zones are present: the central area destined to give the embryo, amnion and allantois, and the peripheral area opaca which will give rise to the yolk sac. Grafting the central area of a quail blastoderm on a chick area opaca results in the development of a quail embryo with a chick yolk sac¹². The lymphoid organs of such chimaeras are entirely populated by quail lymphoblasts or lymphocytes (identifiable by their nuclear marker¹³) in spite of the presence of 95% chick erythrocytes in the circulation with up to 7 d of incubation¹⁴. As there is no quail yolk sac in the system, this proves the existence of an intraembryonic source of stem cells.

The studies of lymphoid organ development which have been conducted in the interspecies system demonstrate that no incompatibility phenomena occur in these experiments and that reticular cells of the lymphoid organ rudiments are attractive towards heterospecific stem cells^{5,6}. However, as quail and chick have different paces of development, this might influence cell interactions. Therefore, we considered it important to study whether similar results were obtained in a histocompatible chick-chick combination, using the sex chromosomal difference as a marker¹⁵.

We transplanted the whole central area of chick embryos on the area opaca of histocompatible embryos *in ovo*. Grafting was carried out at 33–55 h of incubation, the donor and recipient blastoderms being matched for stage in the range of 8–25 somites, that is, before the beginning of active circulation¹⁶. With regard to sex, the grafts were made randomly, resulting in four different combinations, ZZ/ZZ, ZW/ZZ, ZZ/ZW, ZW/ZW. Thus, half of the transplants should be 'sex chimaeras'. About 50% of the grafted 200 embryos developed a successful vascularisation between the two components. Nineteen embryos survived to a final age of 16–19 d of incubation, and were treated with colcemid for chromosome analysis of lymphoid organs. The optimal dose of colcemid allowing a maximal number of metaphases was close to the toxic dose, and only a limited number of embryonic cells was available for final analysis; on the other hand, metaphases were readily available in satisfactory numbers from 2-d-old chicks examined in the later part of this study.

At 16–19 d of development, both the thymus and bursa have long been colonised by stem cells and are highly lymphoid. If the yolk sac were the source of lymphoid stem cells, in about half of the embryos (ZZ/ZW and ZW/ZZ combinations), some dividing cells should belong to the sex opposite to that of the embryo as diagnosed from the gonads. Such is not the case

Table 1 Chromosome analysis of chick embryos grafted on the foreign yolk sac

Organ	Embryos		No. of cells analysed*	
	No.	Sex	♀	♂
Bursa	5	♀	22	0
	7	♂	0	31
Thymus	6	♀	27	0
	7	♂	0	25
Spleen	5	♀	30	0
	7	♂	0	33
Bone marrow	7	♀	48	1
	7	♂	1	67

33–55-h-old chick embryos (Hamburger–Hamilton stage 9–25 with 8–25 somites; ref. 16) were grafted on the age-matched histocompatible yolk sac as described elsewhere¹². White Leghorn Line V (genotype $B^{15}B^{15}$) chick embryos were used. At 16–19 d of incubation (Hamburger–Hamilton stage 42–45), chromosome analysis of cells in the bursa, thymus, spleen and bone marrow were carried out as described by Owen¹⁵.

*Those representing the sex opposite to that of the embryo are italicised.

(Table 1); no cells of discrepant sex were recorded from 13 thymuses, 12 bursas and 12 spleens which yielded identifiable metaphases. In 115 metaphases examined from 14 bone marrows, two—in separate embryos—were deemed to belong to the discrepant sex. If such a low score is accepted as meaningful, these cells may be 'loiterers', remnants of a transitory homing of yolk sac stem cells in the erythropoietic organs of the embryo. Indeed, in the quail–chick system such a transitory homing has been demonstrated in the spleen, at the time when this organ becomes actively engaged in erythropoiesis¹⁴, and a similar process is likely to occur in the bone marrow.

The overwhelming majority of mitoses conforming to the sex of the animal, and the absence of any discrepancy at all in the two primary lymphoid organs, thymus and bursa, show that in the chick–chick system, as in the quail–chick experiments, lymphoid organs are populated predominantly, or even exclusively, by stem cells originating in the embryo, that is, the part developed from the central area, in preference to stem cells from the yolk sac, the component derived from the area opaca.

Table 2 Chromosome analysis of 10 2-d-old chicks transplanted with 10^7 7-d yolk sac cells at 14 d of incubation

Sex of cell recipient	No. of cells of opposite sex/30 mitoses scored		
	Bursa	Thymus	Bone marrow
♀	2	0	2
♀	3	3	2
♀	0	0	3
♀	6	4	3
♀	1	2	3
♂	1	2	1
♂	1	4	5
♂	3	2	2
♂	5	1	5
♂	0	2	2
Total	22	20	28
% donor cells	14.7	13.3	18.7

Pooled male and female cells in 0.1 ml were injected intravenously into the recipients given 850 rads of X irradiation (57 rad min⁻¹) 1 d earlier. The cells were prepared as described elsewhere¹⁰.

Because extensive movements of cells occur through circulation between the embryo and its appendages, we repeated Moore and Owen's irradiation reconstitution experiment² to find out whether we could demonstrate that the 7-d yolk sac contained stem cells capable of replenishing the lymphopoietic system. 10^7 pooled yolk sac cells from 7-d male and female embryos were injected in 14-d histocompatible irradiated embryos; thus all recipients received 5×10^6 cells of the opposite sex. The final calculated percentage of donor cells found in the haemopoietic organs at 2 d after hatching (Table 2) was of the same order of magnitude as that reported by Moore and Owen². Thus, in contrast to the results of the grafting experiment, a number of

cells from the yolk sac home to the thymus, bursa and bone marrow, indicating the presence of stem cells capable of lymphoid differentiation in the 7-d yolk sac.

An important difference between irradiated injected embryos and yolk sac grafted embryos is that, in the former, the haemopoietic system has been depleted by the irradiation. In this case, yolk sac cells, which have been shown to be multipotent in several experiments^{14,17}, find free spaces in the haemopoietic organs, and fill them in the absence of any competition. On the other hand, blood connections have long been established between the yolk sac and the embryo at 7 d of incubation; thus, the lymphoid stem cells present in the 7-d yolk sac may be either cells which differentiated *in situ*, or cells which formed in the embryo proper and travelled through the circulation to the yolk sac.

Our results do not bear on possible earlier events of haemopoietic differentiation at the time of gastrulation. Haemopoietic potentialities at that stage are present at the level of the primitive streak as well as more peripherally¹⁸. This might be the time when precursors of stem cells in the central area of the blastoderm first become committed.

Our results support previous conclusions obtained in a heterospecific system⁷, as well as those based on attempts to reconstitute functionally cyclophosphamide-treated recipients with yolk sac cells^{8–11,19}. The present findings obtained in the chick yolk sac–embryo chimaeras demonstrate the existence of an intraembryonic source of lymphoid stem cells. Furthermore, it is likely that stem cells originally formed in the yolk sac play little, if any, role in the colonisation of lymphoid organ rudiments.

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Appearance of fibronectin during differentiation of mouse teratocarcinoma *in vitro*

THE cell surface glycoprotein known as fibronectin (reviewed in ref. 1) is absent from transformed cells and its distribution changes during morphogenesis, as kidney differentiation has shown². We report here that pluripotential embryonal carcinoma (EC) cells from a mouse teratocarcinoma acquire surface-associated fibronectin as they differentiate into cells with the morphology of endoderm. We also report that the fibronectin is concentrated on the basal lamina-like extracellular matrix of embryoid bodies which develop in culture. This suggests that fibronectin has a function in the organisation of structures during morphogenesis.

Rabbit antiserum³ was raised against human plasma fibronectin and was also reactive against murine cell surface fibronectin⁴. The purity of the antigen as well as the speci-

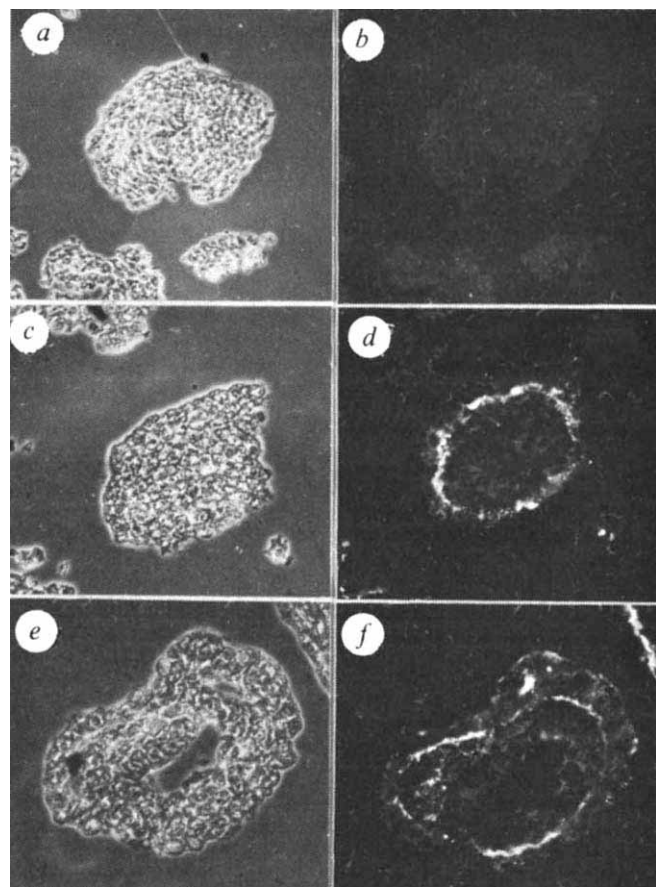


Fig. 1 Developing embryoid bodies stained by indirect immunofluorescence³ for fibronectin. EC (OC15S1) cells were grown for 4 d in tissue culture dishes (Eagle's minimal essential medium+10% heat-inactivated foetal calf serum), and then transferred as aggregates to non-adhesive bacteriological Petri dishes for various times. Cells were fixed at +4 °C for 24 h with a mixture containing 99 parts of ethanol and 1 part of glacial acetic acid, then dehydrated with xylene and paraffin-sectioned according to Sainte-Marie¹⁰. Sections were stained using anti-fibronectin rabbit serum and fluorescein isothiocyanate conjugated sheep anti-rabbit immunoglobulin (Wellcome), and examined in a Zeiss Universal microscope in phase contrast (*a*, *c* and *e*), and by fluorescence microscopy (*b*, *d* and *f*) using an epi-illuminator ($\times 550$). *a*, Aggregate of EC cells after transfer to Petri dish; *b*, aggregate of *a* shows no fibronectin fluorescence; *c*, solid embryoid body 4 d after transfer; *d*, note layer of peripheral fluorescence between endodermal cells and inner embryoid body seen in *c*; *e*, cystic embryoid body 8 d after transfer; *f*, fibronectin fluorescence is characteristically located under peripheral endodermal cells, and lines surfaces of some of the cysts seen in *e*. When anti-fibronectin serum was replaced by normal rabbit serum or anti-fibronectin serum blocked with purified fibronectin, no fluorescence was seen.

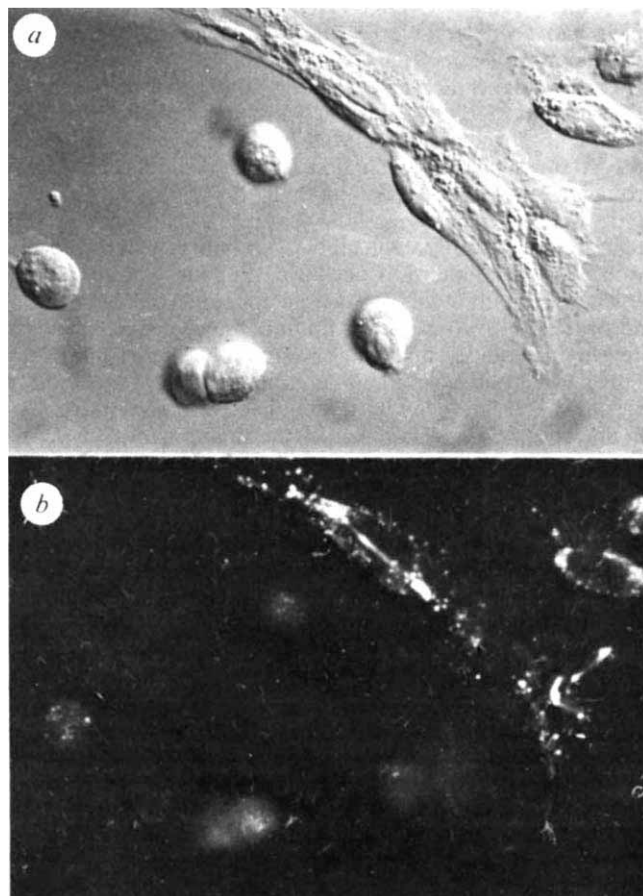


Fig. 2 Differentiating EC cells stained by indirect immunofluorescence for fibronectin. Cells were plated at low density (1×10^6 per 90-mm dish) and grown on glass cover slips in the same medium as in Fig. 1 but in the presence of 0.05 mM dibutyryl cyclic AMP. The medium was changed daily. Cells were fixed for 30 min in 3% paraformaldehyde in 0.1 M phosphate buffer and -20 °C acetone for intracellular detection of fibronectin ($\times 550$). *a*, round EC cells and flattened endodermal type cells after 2 d in culture as seen in Normarski interference contrast microscopy optics; *b*, the same cells in immunofluorescence. Note faint fluorescence in EC cells and strong patchy intracellular and fibrillar extracellular type³ fluorescence in endodermal type cells.

ficity of the antiserum have been documented in detail³. The clone of teratocarcinoma cells used (OC15S1) had been derived from a single cell⁵ and consisted only of pluripotential EC cells. Loosely attached aggregates developed within 4 d after EC cells had been plated on tissue culture dishes. When the aggregates were then grown in suspension culture in non-adhesive bacteriological Petri dishes, embryoid bodies developed. After 3–5 d, the embryoid bodies consisted of a core of EC cells surrounded by a surface layer of endodermal cells^{6,7}, mimicking mouse embryos at the egg cylinder stage, about 5 d of development^{8,9}.

Indirect immunofluorescence staining³ showed that aggregates of EC cells lacked detectable fibronectin (Fig. 1*a* and *b*). But in the solid embryoid bodies, the zone between the peripheral endodermal cells and the internal EC cells stained strongly positive for fibronectin (Fig. 1*c* and *d*). In the next stage of structural development, when cystic embryoid bodies were formed, fluorescence was located under the endodermal cells and partly lined surfaces of the cysts (Fig. 1*e* and *f*).

If EC cells were plated at low density in monolayer cultures, particularly in the presence of dibutyryl cyclic AMP, many of them would differentiate into flat cells resembling the endodermal cells of the embryoid bodies. These cells stained strongly positive for both extracellular

and intracellular fibronectin, whereas the undifferentiated cells had no detectable extracellular and little or no intracellular fibronectin (Fig. 2).

Our results indicate that differentiation of EC cells into endodermal cells is associated with the expression of fibronectin. In developing embryoid bodies a layer of fibronectin forms under the endodermal cells. The position of this layer coincides with the basal lamina in the embryoid bodies^{6,7}. Our monolayer cultures show that EC cells do not produce and retain readily detectable amounts of fibronectin, whereas the endoderm-like cells are the source of fibronectin.

The distribution of fibronectin in embryoid bodies is in accord with the results of immunofluorescence studies of developing mouse² and chick¹¹ embryos. Such studies have shown that fibronectin is characteristically localised in various basal laminae and the connective tissue matrix. The importance of basal laminae in the maintenance of orderly tissue structure is becoming evident¹². Our results suggest that extracellular material which contains fibronectin plays an organising role in morphogenesis. Our findings further show that the appearance of surface-associated fibronectin, which is transformation-sensitive¹, correlates with the development of malignant EC cells into endodermal cells with a differentiated phenotype.

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Note added in proof: We have recently shown¹³ that a layer of fibronectin also appears in the egg cylinder of the early mouse embryo, the normal counterpart of the embryoid body. The expression of fibronectin by pre-implantation mouse embryos and by stem cells of other teratocarcinoma cell lines has similarly been reported¹⁴.

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A human fibrosarcoma cell line producing multiplication stimulating activity (MSA)-related peptides

We suggested previously that transformed cells may produce and release cellular growth factors capable of interacting with a specific class of cellular receptors, making them unavailable as receptors for external ligands^{1,2}. A prediction of this model was that a human fibrosarcoma line, which had been shown not to bind ¹²⁵I-labelled multiplication stimulating activity (MSA)³, might be secreting molecules functionally related to MSA. We describe here some properties of growth factors produced by this human

tumour cell line, derived from a 25-yr-old female with fibrosarcoma of the leg. The cells produce a family of MSA-related compounds with different molecular weights that can be separated on Sephadex G-75. The major activities correspond to proteins of approximate molecular weight (MW) of 11,000 and 7,000. Both of these fractions stimulate cell division, and will compete with ¹²⁵I-labelled MSA for its receptors on mouse, rat or human cell membranes. Fibrosarcoma cells in culture may constitute an important alternate source to human serum and plasma

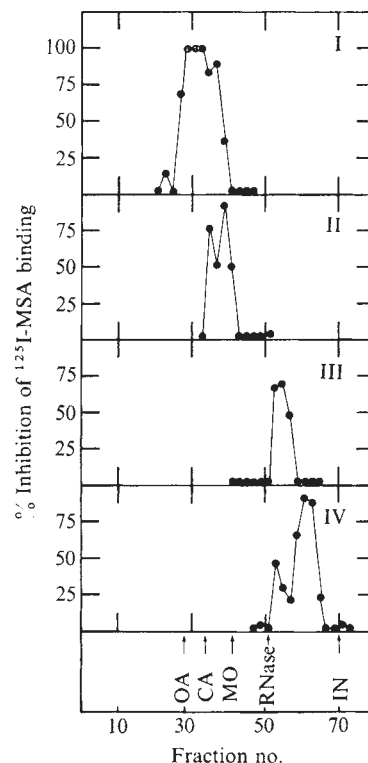


Fig. 1 MSA-competing activity produced by human fibrosarcoma cells. A confluent monolayer of human fibrosarcoma cells was incubated with serum-free Waymouth tissue culture medium³ for 48 h, the medium was clarified of cellular debris by centrifugation and the supernatant fluid was used as the conditioned medium. The MSA competing activity was initially fractionated on a column of Dowex-50 resin⁴. The competing activity from this column was further fractionated on a Sephadex G-150 column (5 × 90 cm) which was developed and eluted with 1 M acetic acid. This column gave four broad peaks (peaks I-IV) of MSA competing activity as measured by the radioreceptor assay. Centre cuts were taken from each of these peaks and rechromatographed on a second acid gel filtration column, Sephadex G-75 superfine (1.5 × 90 cm) to give the data shown. The protein markers were: ovalbumin (OA), 45,000; carbonic anhydrase, (CA), 31,000; myoglobin (MO), 16,900; RNase, 13,800; and insulin (IN), 6,000. The competing activity in the G-75 fractions was determined using a radioreceptor assay on subconfluent monolayers of cell cultures. Approximately 5 × 10⁵ normal rat kidney (NRK) cells were seeded per 35 mm cluster plate well (Costar 3506). The cells were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum 24 h before binding ¹²⁵I-MSA. The cells were washed twice with 2 ml portions of binding buffer which consists of DMEM containing bovine serum albumin (BSA) 1 mg ml⁻¹ and 50 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES), adjusted to pH 6.8. Binding reactions were initiated by the addition of 1 ml of binding buffer containing 1 ng of ¹²⁵I-MSA (11,600 c.p.m.) with or without unlabelled MSA or material from a column fraction. Plates were incubated at 22°C for 90 min. After incubation, the medium was removed and the cell monolayers were washed four times with cold binding buffer to remove the unbound ¹²⁵I-MSA. The amount of radiolabelled MSA bound to the cells was measured after lysing the cells. Radioactivity present in the lysate was determined using a Beckman 7000 γ counter at 67% efficiency. Nonspecific binding was estimated by determining the amount of cell-bound radioactivity in the presence of 1 μg of unlabelled MSA. In these conditions the cells specifically bound 3.5% of the input counts or 405 c.p.m. above the nonspecific binding (75 c.p.m.).

for the isolation and characterisation of growth stimulating factors.

Cells were grown in Waymouth's medium³ in the absence of added protein and the supernatants were collected at 24–48-h intervals. The supernatant fluids were processed as previously described for purification of MSA from a rat liver cell line⁴. Activity was followed using the radioreceptor assay with living cells in culture². MSA competing activity recovered from a Dowex-50 column was lyophilised, suspended in buffer and filtered through Sephadex G-150. Four active fractions were obtained with apparent MWs of 40,000 (peak I), 20,000 (peak II), 11,000 (peak III), and 7,000 (peak IV): the peaks contained respectively 8.7, 6.7, 5.4, and 4.2% of the protein in the conditioned medium. Each of these peaks was pooled and rechromatographed on a Sephadex G-75 column. Aliquots from each fraction were then assayed for competing activity in the MSA radioreceptor assay and the elution profiles of the ¹²⁵I-MSA competing activities are shown in Fig. 1. The first Sephadex G-150 fraction (I), when rechromatographed on Sephadex G-75, gave two peaks of competing activity. The first and main component migrated as molecules between 35,000 and 45,000 MW; this activity, present in fractions I and II, specifically binds ¹²⁵I-labelled MSA and seems to be an MSA-binding protein also secreted by these cells (see below). The second fraction from Sephadex G-150 eluted from Sephadex G-75 with a major peak of activity at approximately 18,000–21,000 MW. The third fraction (III) gave a single peak of competing activity on Sephadex G-75 with MW approximately 11,000 and the fourth fraction gave two peaks of competing activity with apparent MWs of 11,000 and 7,000. Fractions III and IV were chosen for further study because they showed the most cell growth stimulatory activity. The MSA competing activities were heat stable, retaining full activity after heating at 56 °C for

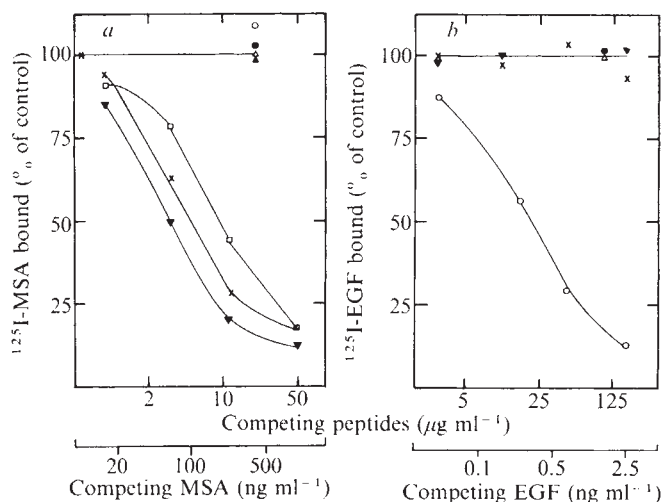


Fig. 2 Radioreceptor competition reactions for ¹²⁵I-MSA and ¹²⁵I-EGF. The radioreceptor assays were performed on live cells growing in attached monolayers as described in Fig. 1 legend (5×10^5 NRK cells per well for the MSA assays and 2.5×10^4 mink lung cells [CCL 64] per well for the EGF assays). Radiolabelled ligands were mixed with various amounts of unlabelled peptides and incubated with the appropriate cells for 90 min at 22 °C as described in Fig. 1 legend. The unlabelled peptides used as competitors are: MSA (□); EGF (○); FGF (●); NGF (△); insulin (▲); fractions III (×) and IV (▼) from the Sephadex G-150 gel filtration chromatography. *a*, Competition for MSA binding to NRK cells using 1 ng of ¹²⁵I-MSA (11,600 c.p.m.) as the radiolabelled peptide. The concentration of competing MSA is shown on the lower scale and the concentrations of the other peptides are on the upper scale. *b*, Competition for EGF binding sites to mink lung cells (ATCC, CCL 64) using 0.2 ng of ¹²⁵I-EGF (14,600 c.p.m.) as the radiolabelled ligand. The concentration of competing unlabelled EGF is shown on the lower scale, and concentrations of all the other competing peptides on the upper scale.

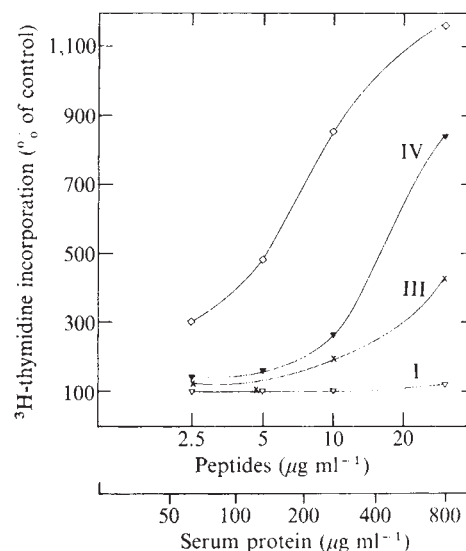


Fig. 3 Stimulation of ³H-thymidine incorporation into DNA in serum-deprived NRK cells by calf serum and fractions of conditioned media from the human fibrosarcoma line. Serum-deprived, subconfluent NRK cells were prepared by replica plating 2.5×10^4 cells in 16 mm Linbro plate wells (FBI6-24TC) in DMEM containing 10% calf serum. After a day the media was removed and replaced with 1 ml per well of Waymouth medium (GIBCO, MD 705/1) containing 0.05% foetal calf serum. Four days later 0.1 ml of binding buffer containing the sample to be tested was added without changing the medium. The additions were: fraction I (▽); fraction III (×); fraction IV (▼); and foetal calf serum (◇). After 16 h the cells were exposed for 8 h to 2.5 μCi of ³H-thymidine (specific activity 20 Ci mmol⁻¹). The medium containing the radiolabelled thymidine was removed, the cultures were washed twice with 1 ml of DMEM containing 100 μg ml⁻¹ of unlabelled thymidine, and incubated for 30 min in the same medium. After incubation, the media was removed, the monolayers were washed again, the cells lysed with 0.5% sodium dodecyl sulphate containing 5 mM EDTA, and the DNA precipitated by adding the lysate to 3 volumes of cold 10% trichloroacetic acid. The precipitated DNA was removed by filtration, the filters dried, added to counting vials with 10 ml of a mixture of toluene and Liquifluor (NEN, NEF 903), and the radioactivity measured in a Beckman LS250 liquid scintillation counter. Control cell cultures incorporated 28,200 c.p.m. of ³H-thymidine. Protein was estimated by the method of Lowry *et al.*¹⁷.

30 min. They were sensitive to protease and disulphide reagents, indicating that the active species in these fractions are proteins with intrachain disulphide bonds required for activity.

The specificity of MSA competing activity was examined. To test for a possible protease activity in fraction III and IV, they were incubated with ¹²⁵I-MSA using the radioreceptor assay conditions, and chromatographed in neutral conditions on a Sephadex G-75 column. The elution profile of the incubated mixtures of ¹²⁵I-MSA and fractions was the same as that for the ¹²⁵I-MSA alone, indicating that the MSA competing activity in these fractions was not a protease that degraded the ¹²⁵I-MSA. To show direct interactions of these competing activities with the cellular MSA receptors, aliquots of fractions III and IV were incubated with cells in binding conditions (see Fig. 1 legend); the fractions were then removed, the cells washed four times with binding buffer, and tested for their ability to bind ¹²⁵I-MSA. In these conditions fractions III and IV blocked the ability of MSA to bind to its specific receptor sites. The specificity of the MSA competing activity was further examined by comparing its ability to compete in the MSA radioreceptor assay with its ability to compete in the epidermal growth factor (EGF) radioreceptor assay². Fractions III and IV tested in an MSA radioreceptor assay (Fig. 2*a*) gave 50% competition, at 7 and 4 μg ml⁻¹ respectively. Pure MSA produced by a rat liver cell line⁵ (provided by M. Rechler

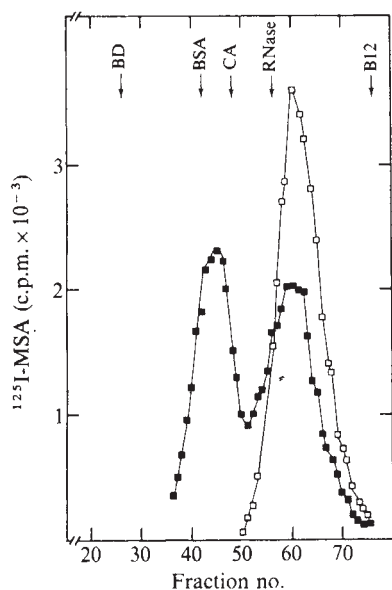


Fig. 4 Sephadex G-150 chromatography of ^{125}I -MSA and ^{125}I -MSA preincubated with an aliquot of fraction I (see Fig. 1). The samples were neutralised and incubated with 0.5 ml of binding buffer (Fig. 1 legend) at 22°C for 1 h and at 4°C for an additional 2 h before being chromatographed on a column of Sephadex G-150 (2.6×35 cm). The column was eluted with 0.14 M NaCl containing 0.01 M BES and 0.001 M CaCl_2 and the pH adjusted to 7.2. Fractions of 35 drops were collected and each fraction counted in a Beckman 7000 γ counter at 67% efficiency. The profiles are: ($\square$), for 5 ng of ^{125}I -MSA (30,000 c.p.m.) and ($\blacksquare$), for 5 ng of ^{125}I -MSA (30,000 c.p.m.) incubated with 1 mg of protein from fraction I.

and P. Nissley, US NIH) showed 50% competition at $0.17 \mu\text{g ml}^{-1}$. Epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF) and insulin were tested at concentrations up to $20 \mu\text{g ml}^{-1}$ in the MSA radioreceptor assay, and none of them competed with MSA binding. Fractions III and IV did not compete in the EGF radioreceptor assay (Fig. 2b), indicating that the competition observed in the MSA radioreceptor assay was specific.

Figure 3 shows the growth stimulatory effect of the fractions when tested for induction of cellular DNA synthesis using rat kidney fibroblasts. As compared to whole calf serum, the fractions from the human cells showed considerable potency. At a fraction IV level of $5 \mu\text{g ml}^{-1}$ there is a fivefold stimulation of DNA synthesis. With calf serum, in the conditions used, $150 \mu\text{g ml}^{-1}$ is required for fivefold stimulation of DNA synthesis. Continued exposure of cells to fraction III or fraction IV in 0.1% calf serum results in a two- to fourfold increase in final cell density of rat kidney or human diploid fibroblasts. The growth stimulatory ability of fractions III and IV relative to their activity in the radioreceptor assay is comparable to that of MSA produced by the rat liver cell line. The elevation of final cell density of normal rat kidney cells treated with either fraction III or IV was comparable with that seen with MSA.

Fractions I and II had little or no growth stimulatory activity even though they compete in the MSA radioreceptor assay (Fig. 3). These fractions seem to contain an MSA-binding protein also secreted by the fibrosarcoma cells. When fraction I was incubated with ^{125}I -MSA and chromatographed at neutral pH on a Sephadex G-150 column, a ^{125}I -labelled MSA binding protein complex can be identified which has an apparent MW of between 50,000 and 60,000 (Fig. 4). This binding activity seems comparable to that of the binding proteins produced by the rat liver cells⁶ and found in human serum⁷.

Several MSA-like growth promoting peptides, including human somatomedin A, somatomedin C, nonsuppressible

insulin-like activity (NSILA-I, NSILA-II) have been isolated from human plasma or serum⁸⁻¹². Studies with factors from human serum or plasma have shown at least two peptides (NSILA-I and NSILA-II) that differ substantially in their overall amino acid sequence^{11,13}. These have been termed IGF-1 and IGF-2; the terminology (insulin-like growth factors) has been suggested to emphasise the growth stimulatory properties of these polypeptides¹³. These peptides are MSA-like in the that they can compete in various radio-receptor assays with MSA⁵ and can stimulate DNA synthesis and cell division¹⁴. The levels of these MSA-related peptides in the plasma have been reported to be associated with final adult size and with the rate of preadolescent growth. Dwarfs of the Laron type are markedly deficient in the production of somatomedins and are clinically unresponsive to treatment with human growth hormone¹⁵. This replacement therapy with certain purified human cell-derived growth factors might be a realistic objective.

Fibroblasts in culture respond to EGF, FGF and the various MSA-like peptides, as well as to other classes of polypeptide hormones in serum, by initiating cell division. The human fibrosarcoma line described here produces MSA-like growth factors that are readily distinguished, using the MSA receptor assay, from the other major classes of growth factors. Whether the cells are making several different primary gene products or whether the various molecular species are different forms of the same compound is not yet clear. Newly developed specific radioimmunoassays for somatomedin A, somatomedin C and NSILA (IGF-1) should help to resolve these questions. Clones of cells are being selected and exposed to mutagens in order to test whether the different molecular species obtained by gel filtration are expressed coordinately in different clones, and whether the synthesis of the binding protein is under independent control from that of the growth factors. Making use of the fact that human chromosomes are preferentially lost from rodent-human somatic cell hybrids it should be possible to localise the chromosomes involved in the production of the growth factors and to purify the structural genes.

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Cytochalasin B inhibits lymphocyte transformation through its effects on glucose transport

THE effects of the fungal metabolite, cytochalasin B, on lymphocyte transformation induced by mitogenic plant lectins have been much studied¹⁻⁷. Results have generally been interpreted in terms of the known effects of cytochalasin B on the microfilaments of the cellular cytoskeleton. A potential site of action which has been largely ignored lies in the inhibitory effects of cytochalasin B on metabolite transport. Cytochalasin B is a potent, competitive inhibitor of erythrocyte glucose transport⁸, and we have shown that it also inhibits thymocyte glucose transport, and that concanavalin A-stimulated glucose transport is more sensitive to this inhibition⁹. Glucose transport has been shown to be rate-limiting for thymocyte glycolysis¹⁰. Also, glucose has been found to be essential for mitogen-stimulated DNA synthesis in lymphocytes¹¹, and we show here that cytochalasin B can exert its inhibitory effect on DNA synthesis by inhibiting glucose transport.

We titrated the proliferative response of rat thymus lymphocytes to concanavalin A with various concentrations of glucose and cytochalasin B (Fig. 1). In the absence of cytochalasin B,

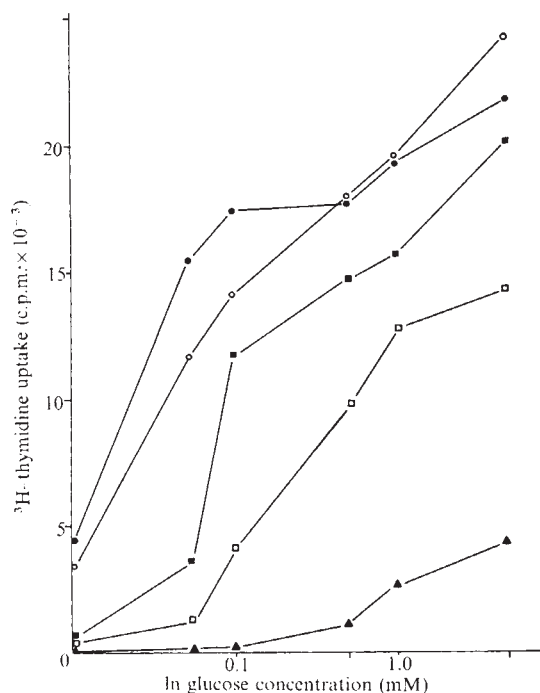


Fig. 1 The effects of glucose and cytochalasin B on ³H-thymidine incorporation into concanavalin A-stimulated rat thymocytes. Rat thymus lymphocytes from male, outbred, 7-8 week old Wistar rats were prepared in sterile phosphate buffered saline³ and cultured in 200-μl microtitre wells at a density of 5×10^6 per ml. The medium was glucose-free Eagle's medium + 15% dialysed horse serum (dialysed for 24 h against five changes of sterile phosphate buffered saline) + 50 μM 2-mercaptoethanol. Concanavalin A was present at a final concentration of 10 μg ml⁻¹. Cytochalasin B was maintained as a stock solution at -20°C (20 mM) and was diluted in phosphate buffer on the day of use. The final concentration of dimethyl sulphoxide (used in the cytochalasin stock solution) in the cultures was less than 0.1%. After 24 h, ³H-thymidine was added to each well, and the cells were collected on filter paper (using a Skatron automatic harvester) after a further 16 h, and counted for radioactivity. The results are the average of three experiments, each performed in triplicate, in which the pattern of the response was the same on each occasion. Background ³H-thymidine uptake was around 200 c.p.m. The graphs show the effect of glucose in the presence of concanavalin A plus (●), no addition; (○), 0.1 μM cytochalasin B; (■), 0.5 μM cytochalasin B; (□), 1.0 μM cytochalasin B; (▲), 5.0 μM cytochalasin B.

the effect of glucose was biphasic, while little DNA synthesis was observed in the absence of added glucose. Very low concentrations of glucose (50 μM) caused a substantial stimulation of ³H-thymidine uptake, and a further smaller stimulation was observed as the concentration of glucose became saturating for the glucose carrier ($K_m = 0.3$ mM, ref. 11). The effects of cytochalasin B were clearly dependent on the glucose concentration in the medium. In the absence of glucose, or at low concentrations, cytochalasin B was inhibitory at all concentrations tested. A stimulatory effect of low concentrations of cytochalasin B^{2,7} was only observed at supersaturating glucose concentrations. From Fig. 1 it is clear that glucose can overcome the inhibitory effects of cytochalasin B, as one would expect if the inhibition of glucose transport by cytochalasin B were competitive. Strangely, studies in lymphocytes¹³ have failed to observe competitive binding of glucose and cytochalasin B to high affinity plasma membrane sites. This has been observed in erythrocytes⁸, and the concentrations of cytochalasin B which inhibit lymphocyte glucose transport⁹ are close to the reported affinity of the erythrocyte glucose carrier for cytochalasin B.

Glucose is probably required in lymphocyte proliferation for the synthesis of precursors. The *de novo* synthesis of purines and pyrimidines may be essential for lymphocyte proliferation¹². Similarly, cytochalasin B induced inhibition of phospholipid metabolism in mitogen-treated lymphocytes may result from a lack of α-glycerophosphate due to an inhibition of glycolysis³. It is particularly interesting to note that lymphocytes escape the inhibitory effects of cytochalasin B within a few hours of addition of the mitogen^{3,7}.

In conclusion, our results suggest that greater care should be taken in interpreting the effects of cytochalasin B on lymphocyte transformation, which may be primarily due to effects on metabolite transport. This caution may be applied to the many other systems in which cytochalasin B has been used, and in which glucose is an important source of energy, or precursors.

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Association between an HLA haplotype and low responsiveness to tetanus toxoid in man

THE major histocompatibility complex (MHC) in man, the *HLA* gene complex, has been assumed to code not only for histocompatibility antigens but also for immune responsiveness and control of disease susceptibility¹. Although several attempts have been made to delineate the existence of *HLA*-linked immune response genes (*Ir* genes) in man, the data are controversial²⁻⁶. We demonstrate here a striking association between a particular *HLA* haplotype and low responsiveness to tetanus toxoid *in vitro*, which suggests that immune suppression genes exist in man.

Ninety-two Japanese premedical students were given a primary intramuscular immunisation with tetanus toxoid (5 LF of the

alum-precipitated form). Peripheral blood was drawn before and 2 weeks after immunisation, and lymphocytes were prepared by Ficoll-Conray gradient centrifugation. They were washed and resuspended at 1×10^6 cells ml^{-1} in RPMI-1640 with 2 mM L-glutamine, 10% pooled inactivated human sera, 100 units penicillin and 100 μg streptomycin per ml. The *in vitro* immune response of 1×10^5 or 2×10^5 cells to purified tetanus toxoid was tested in triplicate in 0.2 ml medium in flat bottomed microtitre trays (Linbro). Before use, purified tetanus toxoid was passed through a Sephadex G-25 column with phosphate-buffered saline. The cells were incubated with 1 μg or 10 μg of the toxoid in humidified 5% CO_2 and air at 37 °C for 7 d. One μCi of tritiated thymidine (New England Nuclear; specific activity 6.7 Ci mmol^{-1}) was added to each well 16 h before collection. Cells were collected on glass fibre filter paper and the incorporation of ^3H -thymidine measured in a liquid scintillation counter (Aloca).

HLA-A and B typing was performed by the standard microdroplet lymphocyte toxicity test⁷ using antisera of Japanese regional and Kyushu local origin. In order to type rare antigens among Japanese individuals, antisera obtained through exchange from Drs P. I. Terasaki, J. Dausset and D. Kayhoe were used. HLA-D typing was performed by the mixed lymphocyte reaction (MLR) test using *HLA-D* homozygous typing cells.⁸

The immune responsiveness to tetanus toxoid before and 2 weeks after immunisation is shown in Fig. 1. Before immunisation, the histogram of thymidine uptake showed an approximately $\ln$ distribution. Two weeks after immunisation, it showed a clear bimodal distribution indicating that there were low and high responders to tetanus toxoid. The bimodality was most clear at 1×10^5 cells with 1 μg purified tetanus toxoid per well. The mode of the low responders did not shift from the mode of the $\ln$ distribution obtained before immunisation, possibly indicating the presence of non-responders rather than low responders. The mode of high responders to tetanus toxoid seems to fit the tailing of log-normal distribution obtained before immunisation. This suggests that several individuals had received tetanus toxoid immunisation before this study, for a previous study⁹ has shown that persons who receive prior immunisation respond extremely vigorously to tetanus toxoid *in vitro* 2 weeks after the planned immunisation.

Because the amount of thymidine incorporated was distributed bimodally, it was possible to identify, out of 92 individuals, 14 (15.2%) low responders and 78 (84.8%) high responders. Previous data have shown that this high or low responsiveness is antigen-specific⁹.

All 92 individuals were then typed for their HLA-A and B specificities. There was no distortion in HLA antigen frequencies in this population compared with the random Japanese population. The 78 high responders did not show any association with a particular HLA specificity. Low responders, on the other hand, showed a significant association with HLA-B5 (Table 1). Of 14 low responders, 8 (57.1%) had HLA-B5 whereas 22 (28.2%) out of 78 high responders had this specificity ($\chi^2 = 4.52$). In the Japanese population, the new HLA-D specificity, DHO, which was first reported to be in linkage disequilibrium with HLA-Bw35 (ref. 10) is now clearly shown to have linkage disequilibrium with B5 (ref. 11). HLA-D specificities of 12 low and 62 high responders were, therefore, determined by the MLR using two *HLA-DHO* homozygous typing cells. Of 12 low responders, 7 (58.3%) had HLA-DHO, whereas only 7 of 62 high responders (11.3%) had

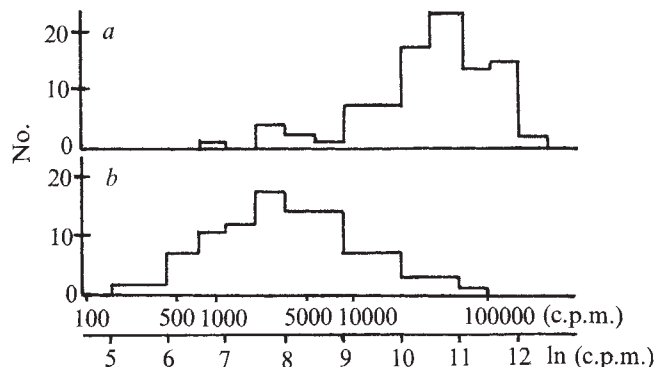


Fig. 1 Immune response to tetanus toxoid measured by antigen-specific T cell proliferation *in vitro*. a, Post-immunisation (1 μg) ($N = 92$); b, pre-immunisation ($N = 92$).

this HLA-D specificity ($\chi^2 = 14.50$). This increase in frequency of HLA-DHO in the low responder group is also highly significant compared to its frequency in the random Japanese population ($\chi^2 = 12.78$). Thus, low responsiveness to tetanus toxoid showed a stronger association with HLA-DHO than with HLA-B5. HLA-B5 is also in linkage disequilibrium with HLA-A9 in the Japanese population¹². In the low responder group, HLA-A9 was slightly increased in frequency (71.4%) compared with that in the high responder group (60.3%), but this association is not statistically significant.

It is apparent that HLA-DHO had the strongest association with low responsiveness to tetanus toxoid, and that the association observed between HLA-B5 and low responsiveness was due to the linkage disequilibrium between B5 and DHO. In this regard, the haplotype frequency (H_f) of *HLA-A9-B5-DHO* calculated by the method described by Piazza¹³ in the low responders was extremely high ($H_f = 0.31$).

Because *HLA-D* codes for the antigens which elicit the MLR (refs 14, 15) this locus has been assumed to be comparable to the *I*-region of the murine *H-2* complex, where *Ir* genes and immune suppression genes (*Is* genes) are located^{16, 17}. The possibility exists, therefore, that the low responsiveness to tetanus toxoid in man might be controlled by either an *Ir* gene or an *Is* gene in strong linkage disequilibrium with *HLA-DHO*. If responsiveness to tetanus toxoid was under the control of an *Ir* gene, the condition of low responsiveness could occur only when this *Ir* gene was absent. The low responders should, therefore, be homozygous at this locus. MLR among *HLA-DHO* homozygous typing cells and low responders to tetanus toxoid, however, showed that all but one low responder who had HLA-DHO were heterozygous for this D antigen. Therefore, if the condition of low responsiveness is under genetic control, it could not be determined by *Ir* genes, but might be controlled by dominant *Is* genes¹⁸. De Vries *et al.* have also suggested the possibility of the existence of *Is* genes in man by showing an association between low responsiveness to vaccinia virus and HLA-Cw3 (ref. 19).

In conclusion, the response to tetanus toxoid in man measured by the antigen-specific T cell proliferation *in vitro* showed clear bimodal distribution in a healthy population. Low responsiveness to tetanus toxoid had significant association with HLA-B5 and stronger association with HLA-DHO, which is a new HLA-D

Table 1 Association between low responders to tetanus toxoid and HLA specificities

HLA type	Low responders	High responders	Control (Japanese)	Low to high	χ^2 value Low to control
	14/92 (15.2%)	78/92 (84.8%)	416		
HLA-A9	10/14 (71.4%)	47/78 (60.3%)	241/416 (58.0%)	0.63	1.02
HLA-B5	8/14 (57.1%)	22/78 (28.2%)	141/416 (34.0%)	4.52*	3.23
HLA-DHO	7/12 (58.3%)	7/62 (11.3%)	11/69 (15.9%)	14.50†	12.78‡

* $P < 0.05$.

† $P < 0.0002$.

‡ $P < 0.002$.

specificity found in the Japanese population. This is the first description of strong association between HLA-D specificity and immune responsiveness in man.

The purified tetanus toxoid was obtained from the Department of Public Health, Massachusetts.

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Fluoridation and mortality due to heart disease

STANDARDISED death rates in the US, due to ischaemic heart disease stopped increasing and began to decrease during the period in which large numbers of cities began fluoridating their water supplies but no notice has been taken of this effect. Gordon and Thom¹ note that deaths attributable to ischaemic heart disease had been increasing until about 1960. By 1968, a clear decrease had begun, and continued until their last observations in 1972. They felt that this decrease (7.3%) could be partially explained on the basis of less severe influenza epidemics, which are thought to increase the mortality rates due to heart disease in the winter months. However, artificial fluoridation was started in 1952, and by 1969 had covered 74 million people². Therefore, the possibility exists that fluoridation might be involved in the declining rates. Ideally, this would be checked by comparing the rates for ischaemic heart disease between fluoridated and non-fluoridated populations. The largest political units in which the distinction between fluoridated and non-fluoridated populations is clear-cut are cities; unfortunately, ischaemic heart disease is not tabulated separately for cities in the Vital Statistics publication. Ischaemic heart disease is an important part of the category 'all heart disease', and those data are readily available. We report here on changes in the more inclusive category (all heart disease) and fluoridation status, which support the possibility that fluoridation is associated with the decreasing rates.

The cities compared were the same as those used for earlier work on cancer mortality rates³. In addition to the 10 fluoridated and 10 non-fluoridated cities which others have studied⁴⁻⁶, I have included 15 other cities. Of these, 10 are the next largest fluoridated cities, with at least 13 yr of fluoridation before 1970.

Table 1 Standardised mortality ratios: heart disease (standard rates, US 1950)

Cities	1949-50	1959-60	1969-70	Decrease 1950-70
10 Original controls	1.079	0.987	0.905	0.174
10 Original fluoride	1.274	1.176	1.033	0.241
5 Extra controls	0.935	0.801	0.794	0.141
10 Extra fluoride	1.042	0.933	0.847	0.195
All 15 controls	1.053	0.944	0.874	0.179
All 20 fluoride	1.221	1.116	0.983	0.238

Five are the non-fluoridated cities which Burk and Yiamouyianis⁷ excluded in their selection of the 10 non-fluoridated controls. The second set of 10 fluoridated cities is important, because it is more comparable to the controls in population growth and initial standard mortality ratios (SMRs) than was the original set of 10 fluoridated cities. Changes in age, sex and race are controlled indirectly by the use of the SMRs, because the detailed data needed for the direct method of adjusting for changes in age, race and sex are not readily available. The data for the SMR calculations were restricted to census years and the year immediately preceding (unless otherwise stated), in order to provide accurate population figures.

The SMRs for deaths due to heart disease are shown in Table 1. There is a 15-20% decrease in the SMRs for each of the four groups over 1950-70. This decrease in SMRs for the fluoridated cities is greater than the decrease for the control cities. The magnitude of the 1950-60 decrease is also greater in the fluoride groups except when compared to the five-city control group. The large drop in the SMRs for the five-city control

Table 2 Standardised mortality ratios: heart disease (standard rates, US 1950, 1960 and 1970 for respective years)

Cities	1949-50	1959-60	1969-70	Decrease 1950-70
10 Original controls	1.077	1.043	1.054	0.023
10 Original fluoride	1.274	1.255	1.219	0.055
5 Extra controls	0.931	0.850	0.928	0.003
10 Extra fluoride	1.037	0.979	0.928	0.055
All 15 controls	1.050	0.999	1.019	0.031
All 20 fluoride	1.219	1.185	1.154	0.065

group over 1950-60 is due almost entirely to the inclusion of Phoenix, Arizona, which experienced a four-fold increase in population in that time. For 1949-50, the number of observed deaths was 63,616 in the 15 control cities and 137,159 for the 20 fluoridated cities, making the standard errors 0.396 and 0.270%, respectively. The standard errors of the difference from 1950-70 are 0.526 and 0.380%, respectively, making the standard error of the difference in change 0.65%. The initial SMRs are considerably higher for all 20 fluoridated cities, so the absolute decrease may be overstating the case, but the relative decreases of 17.0 and 19.5% still leave a 2.5% greater decrease in the fluoridated cities.

The heart disease classifications included in the vital statistics summaries have been modified over this 20-yr period, and these changes might influence the magnitude of the overall decrease. For this reason, SMRs were also calculated using the 1960 US

Table 3 Standardised mortality ratios: all causes (standard rates, US 1950)

Cities	1949-50	1959-60	1969-70	Decrease 1950-70
10 Original controls	1.032	0.922	0.864	0.168
10 Original fluoride	1.092	0.980	0.895	0.197
5 Extra controls	0.997	0.799	0.802	0.195
10 Extra fluoride	1.022	0.927	0.847	0.175
All 15 controls	1.025	0.892	0.846	0.179
All 20 fluoride	1.076	0.967	0.882	0.194

rates for the 1959–60 SMRs and the 1970 US rates for the 1969–70 SMRs. The results of these calculations are shown in Table 2. The 1950–70 comparisons show a 3.1% greater decrease in the fluoridated cities than in the controls.

The SMRs for death from all causes, not just heart disease, are shown in Table 3. It also shows a 15–20% decrease in the SMRs over 1950–70. Considering all 20 fluoridated and all 15 non-fluoridated cities, the decrease was 1.48% greater in the fluoridated cities. In 1949–50, the observed number of deaths in the control cities was 176,252; for the fluoridated cities it was 343,724. The standard error of the difference in decrease was 0.41%. The observed number of deaths due to heart disease in the fluoridated cities was $8,148 \pm 950$ less than expected from the experience in the control cities. It was $5,296 \pm 1,467$ less for all causes of death.

Table 4 US age, sex and race rates adjusted by the direct method per 100,000 population*

	1950	1960	% Decrease	1970	% Decrease
All causes	841.5	760.9	9.6	730.9	13.1
Heart	307.6	286.2	7.0	262.3	14.7

*National Center for Health Statistics (1974) (ref. 8).

The marked decreases in the death rates due to all causes and the rates for heart disease agree well with published rates for the US as a whole (Table 4). These data are rates per 100,000 directly adjusted for age, sex and race rather than by SMR. The control cities in Table 2 showed a 3.1% decrease for heart disease relative to the US as a whole for 1950–70. Adding this value to the 14.7% decrease for the US, shown in Table 4, gives 17.8%, which is essentially the same value (17.9%) as for controls during this period estimated by the indirect method (Table 1). The comparable values for the fluoridated cities is 21.2% and 23.8%. As less than half the population of the US were receiving natural or artificial fluoridation by 1970, the comparison for the fluoridated cities would be expected to be in the observed direction. Therefore, from these data, there is little difference between SMR and direct method of standardisation. The decrease in total mortality rates and heart disease for the US over 1950–60 runs counter to the increasing ischaemic heart disease death rates in that period. Presumably, the mortality rates associated with some heart diseases, like rheumatic disease, were decreasing very rapidly due to antibiotic use at that time.

The present data are consistent with a hypothesis that decreasing mortality rates due to ischaemic heart disease are due to fluoridation. Fluoride might cause a reduction in ischaemic heart disease by inhibiting calcification, for calcification around the area of infarction is thought to be the basis of cardiac imaging with technetium⁹. Intermediate levels of fluoride inhibit soft tissue calcification *in vitro*^{10,11} and are associated with less aortic calcification *in vivo*¹². Less calcification might result in higher free phosphate concentrations in the mitochondria, which are important in maintaining adequate ATP levels and hence cell survival. Thus, while the hypothesis at this point is only an intriguing possibility, it seems worthwhile to obtain the additional epidemiological and laboratory data needed to test the possibility.

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Reversal of recruitment order of single motor units produced by cutaneous stimulation during voluntary muscle contraction in man

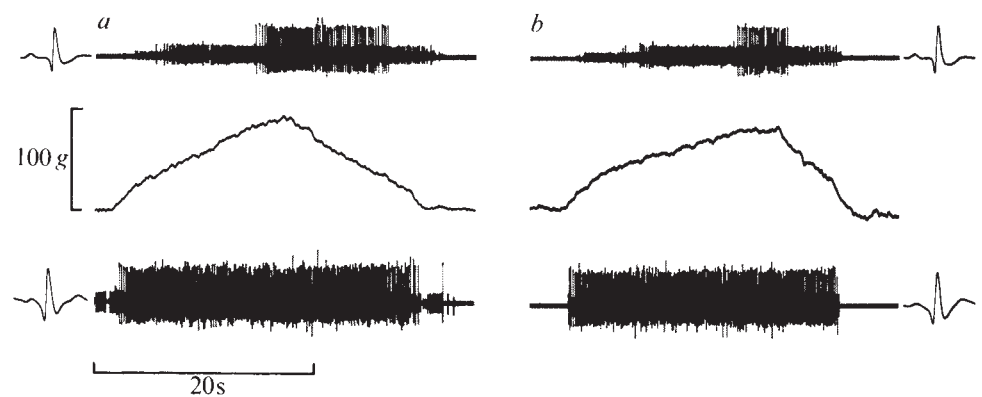
THE strength of a voluntary muscle contraction is determined by the number of motor units active and their discharge frequency¹. As voluntary effort is increased, increasing numbers of motor units become active in a repeatable sequence². Although some exceptions have been reported^{3–8} most of the experimental evidence in animals and man supports the view that this sequence is fixed and invariable^{9–13}. We show here, however, that the order of motor unit recruitment can in fact be changed simply by stimulating cutaneous afferents. Of a sample of 41 motor units (eight experiments, five subjects) all but two units had their recruitment threshold changed by cutaneous stimulation. Examples in which such an effect was sufficient to reverse the normal order of recruitment of two units recorded simultaneously have been found in every subject tested (12 pairs of units, eight experiments, five subjects).

Figure 1a shows a typical recording of the firing pattern of two motor units during voluntary contraction of first dorsal interosseous muscle (1DI). The hand was carefully immobilised and the force of abduction of the index finger produced by contraction of 1DI recorded using a strain gauge positioned against the lateral side of the first interphalangeal joint. Motor unit electrical activity was recorded using two conventional monopolar concentric EMG needles inserted into the muscle. As the subject gradually increased his force of contraction, first the lower threshold unit (1) (Fig. 1, lower trace), then the higher threshold unit (2) (Fig. 1, upper trace) became active. Unit (2) was recruited at a contraction strength $15 \times$ higher than that for unit (1). The difference in threshold for these two units was 75 g, approximately 3% maximum voluntary contraction strength. Repeated trials produced the same result. The subject was unable to contract his muscle in such a way that the higher threshold unit fired alone. The order of recruitment for this pair of motor units seemed, therefore, to be fixed and invariable. This, however, turned out not to be the case, as is shown in Fig. 2.

At the start of Fig. 2a, the subject made a contraction such that unit (1) fired alone. He then increased his force of contraction and unit (2) also became active. Cutaneous stimulation was then started. The digital nerves of the index finger were stimulated continuously at 3 times the threshold for perception (pulse width 50 μ s, 50 shocks s^{-1}) through ring electrodes placed around the finger on either side of the distal interphalangeal joint. This type of stimulus elicits a sensation which is subjectively similar to having the index finger gripped by rough sandpaper. Gradually, unit (1) reduced its firing until unit (2) was firing alone. The end of this period is shown in Fig. 2b, which started 150 s after the beginning of stimulation. Unit (1) stopped firing and the subject was able to maintain unit (2) active alone, at a contraction strength lower than the recruitment threshold for this unit in control conditions (compare Fig. 1 and the last part of Fig. 2a). At the end of the period shown in Fig. 2b, the subject was asked to increase his voluntary effort briefly. Unit (1), which had been recruited before unit (2), then fired only if the subject maintained a contraction greater than that at which unit (2) fired alone. The recruitment order was thus reversed. The subject was quite unable to contract his muscle such that the previously relatively low threshold unit (1) fired alone.

Stimulation was then discontinued, and over a period of several minutes, the control pattern of recruitment returned.

Fig. 1 The recruitment of motor units in first dorsal interosseous muscle during gradually increasing voluntary contraction. The lower and upper traces were obtained using separate intramuscular needle electrodes, and show the activity of the lower threshold motor unit (1) and higher threshold motor unit (2), respectively. In each recording the action potentials of the individual units stand out from the background activity and each has a characteristic shape. An example has been selected from each trace and is shown to one side on an expanded time scale. Middle traces show force of abduction of the index finger. *a*, Control recruitment pattern; *b*, the same pattern recorded 15 min after the end of continuous stimulation of the digital nerves of the index. By spike-triggered force-averaging^{12,19}, twitch amplitude and contraction time for units (1) and (2) were 1 g, 89 ms and 1.9 g, 70 ms, respectively. Vertical force calibration 100 g (approximately 4% maximum voluntary contraction strength).



In Fig. 2*c* (35 s after the end of stimulation) unit (1) started to fire with unit (2). Notice that throughout the record the force exerted by the subject was falling gradually. The subject no longer needed to increase his force of contraction to make units (1) and (2) fire together (compare with last part of Fig. 2*b*); the force needed to fire unit (1) was by now lower

than in Fig. 2*b* but still higher than that in the control. Gradually, however, the effect of the stimulus seemed to wear off, as shown by the fact that unit (1) increased its firing rate in spite of the fact that the force exerted by the subject was decreasing. Two minutes after the end of stimulation (Fig. 2*d*), unit (2) began to reduce its firing frequency and eventually stopped.

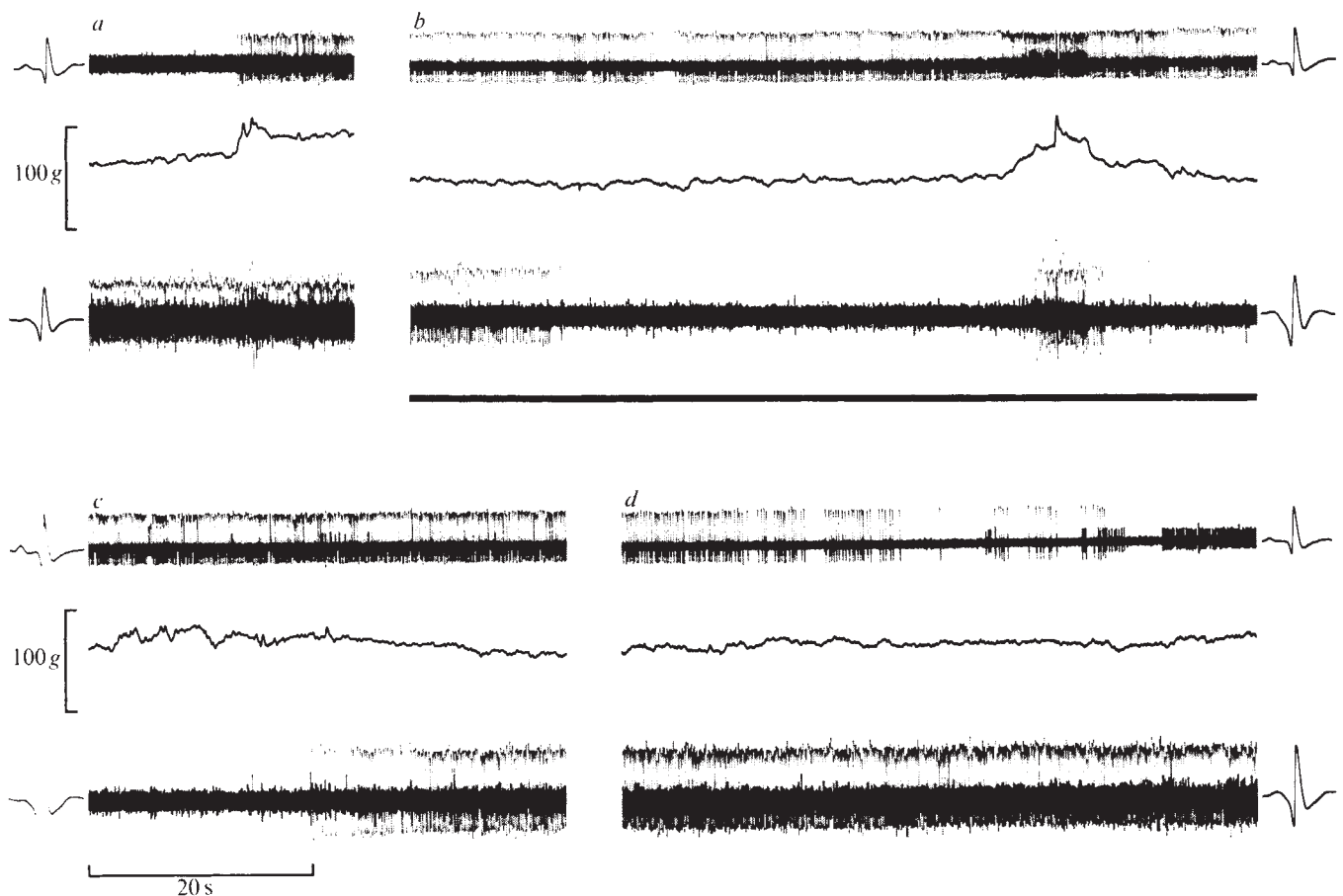


Fig. 2 Reversal of the recruitment order of two motor units in first dorsal interosseous muscle produced by electrical stimulation of the digital nerves of the index finger. Traces selected from a continuous recording, subject contracting steadily throughout. Same motor units and arrangement of traces as in Fig. 1. *a*, Subject first maintained a contraction such that only unit (1) fired; then a stronger contraction when both units (1) and (2) fired together. Unit (2) (upper trace) could not be made to fire alone. *b*, Recording obtained while digital nerves of index stimulated at 3 times threshold (50 p.p.s.). Unit (2) could be made to fire alone. The recruitment order was reversed. As shown at end of trace, unit (1) only then fired if the subject increased his force of contraction above that associated with the firing of unit (2). Single action potential of unit (1), to the right of lower trace, was selected from the burst of activity associated with this period of increased voluntary contraction. Cutaneous stimulation was discontinued 35 s after end of trace *b*. In *c* and *d* restoration of normal recruitment order; *c*, recorded 35 s and *d*, 120 s after end of cutaneous stimulation. In *c* unit (1) became active again with unit (2). In *d* unit (1) could be made to fire alone (compare with *b*). Vertical force calibration 100 g (approximately 4% maximum contraction strength).

Unit (1) could again be made to fire alone at a level of contraction strength which previously, during stimulation (Fig. 2b) and during the earlier part of recovery (Fig. 2c and start of Fig. 2d), had been associated with the firing of unit (2). In contrast to the situation shown in Fig. 2b and c, the subject could no longer make a contraction such that unit (2) fired alone. The control pattern of recruitment had therefore returned. During a gradually increasing voluntary contraction recorded 15 min after the end of stimulation, units (1) and (2) became active in the same sequence as at the start of the experiment (compare Fig. 1a and b).

Some functional significance has previously been sought for the fact that most muscles are very heterogeneous with regard to motor unit mechanical properties¹⁴. Particularly attractive, for example, has been the idea that fast and slow twitch motor units, each with their distinctive muscle fibre types and biochemical specialisation, might be involved selectively in different types of movement^{6,15,16}. This, however, has yet to be demonstrated convincingly. In spite of the apparent functional advantages that might result, the order of motor unit recruitment has so far proved to be disappointingly inflexible¹³. Indeed, its very inflexibility in various different conditions in the hind limb of the decerebrate cat, has led Henneman *et al.* to conclude that the order of recruitment is simply determined by differences in the shape and size of motoneurons^{9,10}. An attractive consequence of this hypothesis is that no specialised distribution of the input to a motoneurone pool need be supposed to account for the orderliness of recruitment. A simple concept for the organisation of synaptic input to the pool is envisaged, namely that every motoneurone shares qualitatively identical inputs. According to this view, the motoneurone pool would seem to be organised in such a way as to prevent, rather than allow, the selective activation of different subpopulations of motor units. It seems clear from the present results, however, that the motoneurone pool of 1DI is not organised in this way, because in the special conditions of our experiments, the recruitment order was changed. The potential seems to exist for the selective activation of different populations of motor units during voluntary contraction of this small intrinsic hand muscle. The result is particularly striking because it shows for the first time that the same steady level of voluntary contraction strength can be maintained by the activity of different populations of motor units. That this effect can be produced by simple cutaneous stimulation is entirely consistent with the experiments of Burke *et al.*^{17,18}, who showed that stimulation of the cutaneous sural nerve in the cat produces predominantly excitatory postsynaptic effects in some medial gastrocnemius motoneurons, has mixed excitatory and inhibitory effects on others, and predominantly inhibitory effects in the remainder. This latter group was found to innervate slow twitch muscle fibres. On this basis alone, one would expect early recruited type S units to be selectively inhibited by skin stimulation. This has now been partly verified experimentally during reflex contractions of cat gastrocnemius⁸. A similar non-homogeneous distribution for the synaptic effects of cutaneous stimulation on 1DI motoneurons in man presumably underlies the change in recruitment order we observed.

Taking the present results together with earlier observations in man on the effects of muscle vibration on recruitment during the initiation of voluntary contraction³, it seems that the order of motor unit recruitment is not in fact invariable, but can be influenced by afferent input. Here, perhaps, lies the significance of our results in functional terms. The first dorsal interosseous muscle is used in manipulative tasks. The fingers daily come in contact with many different sorts of objects, each giving rise to cutaneous afferent input of various types and intensity. The functional advantage of having separate access to different populations of motor units, depending on the nature of associated cutaneous afferent input, now remains to be determined.

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Long-term retention of single and multistate prismatic adaptation by humans

SENSORY rearrangement techniques have been used to study the way in which primates reach accurately for objects. When an object is viewed through wedge prisms, for example, reaching is disrupted but rapidly corrected when the subject is given immediate feedback concerning the position of his pointing limb relative to the target¹. Such adaptation to distorted input is conventionally regarded as a process of minimising the discrepancy between two or more sensory channels (for example, vision and proprioception), with the neglected or non-dominant channel conforming to the position information provided by the other. The role of learning in this process is unclear. When the prisms are removed, the effects of such adaptation disappear rapidly (unlike most learning phenomena), and after an interval of 24 h during which the subject reaches 'normally', most investigators assume² that all traces of adaptation have been eliminated from the nervous system. This view has been challenged recently by results we obtained with squirrel monkeys³, which retained their adaptation over periods often considerably longer than the 48 h spent in their home cages. We also found that they could adapt to two prisms displacing in equal and opposite directions, and eventually conserve both adaptations together with their normal reaching behaviour. Their progressive elimination of error (due to prismatic distortion) as a function of the number of occasions on which they were exposed to prism conditions suggests, furthermore, powerful motor learning mechanisms at work in the adaptation process. We now report similar findings with human subjects.

Our subjects were 34 students at Edinburgh University. Those with corrected vision continued to wear spectacles or contact lenses throughout the experiment. The subjects looked through a wedge prism with their dominant eye and pointed at the target with their preferred hand. The apparatus was an Imhof stand with a circular window at eye level through which the subject could see a target board calibrated in units for 1 cm, but with no numbers visible to the subject. A prism holder, mounted at the rear of the window, allowed the experimenter to slide either of two wedge prisms of 30 diopters or a glass block

into position across it, or occlude it altogether. A white plastic target 1.5 cm high was used with a magnetic base which allowed it to be easily transposed by the experimenter. A panel prevented subjects from seeing their pointing limb until the terminus of their reach. An additional panel could also be fitted to eliminate all visual feedback. Subjects pointed at a total of five different target positions in a sequence determined at random. The index finger of each subject was marked with a dyed stripe to permit easy measurement of error. Following a ballistic response to the target, no corrections under visual guidance were permitted. Instead the subject returned his hand to his lap. The inter-trial interval was 10 s.

All subjects were given a practice session with the glass condition before the experiment proper. This 'pre-exposure' test provided a record of their pointing accuracy under normal conditions measured by their absolute errors. Subjects were divided into two main groups. In group 1 (6 subjects), training was given on one prism only for 5 separate training sessions each of 40 trials. Each session was separated by an interval of 3 d. At the end of training, retention tests were given after an interval of 2 weeks and then of 4 weeks. Apart from tests of reaching with the prism in position, tests were also conducted with a clear glass window to measure 'after effects'. During these tests no visual feedback was given. All 6 subjects were given such tests at the end of each retention test period. By way of 'probe' tests, two of the subjects were also given 'after effect' tests (without feedback) at the beginning and end of each training session. The errors recorded were in the direction of the displacement when the prism was present and in the opposite direction on the 'after effect' tests. To simplify presentation and make direct comparison between both phases of the experiment easier, both sets of errors are given 'positive' values.

Figure 1 shows that all subjects improved dramatically over the series of training sessions, even though each session was separated by an interval of 3 d; by session 5, virtually no error was recorded. The plot of 'first trial' errors per session shows, furthermore, that subjects learnt to predict the correct response, and not merely to adapt faster following a mistake. Figure 1 also indicates excellent retention of this adaptation over periods of 2 and 4 weeks, respectively. The 'after effect' errors, recorded with clear glass and no visual feedback, remain constant and are of considerable magnitude. These results clearly show, therefore, that perceptual adaptation need not be a transient phenomenon. Like the monkey, the human seems to

Fig. 1 Mean (absolute) errors for the first 10 trials of each session (corrected for normal reaching errors) and first trial errors, for 5 training sessions and 2 retention tests (after 2 and 4 weeks) for Group 1 (6 subjects). The mean and first trial errors for the after effect (AE) tests are also shown for the 2 retention tests. The errors recorded with the prism present were in the opposite direction to the after effect errors. $\times$, Prism mean; $\circ$, prism first trial; $\blacktriangle$, glass (AE) mean; $\blacksquare$, glass (AE) first trial.

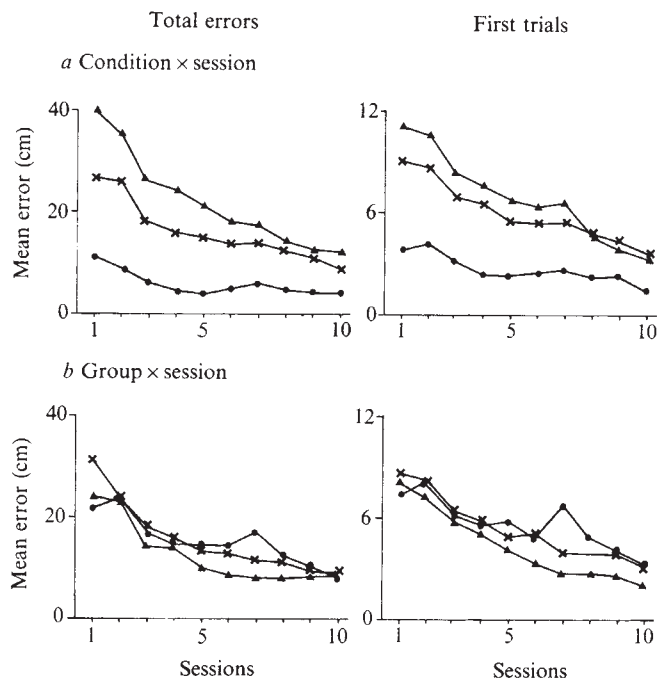
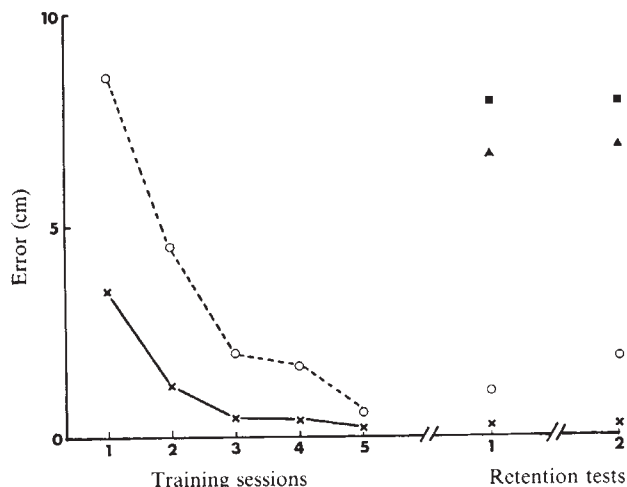


Fig. 2 Mean total (absolute) errors (corrected for normal reaching errors) and first trial errors per session (a) for each condition, ($\times$, 1st prism; $\blacktriangle$, 2nd prism; $\bullet$, glass). Condition $\times$ session interaction, and (b) for each group, Group $\times$ session interaction, for which subjects were given repeated training on each of 3 conditions of prism base left (Pa), prism base right (Pb) and plain glass (G) for 10 sessions. The order of presentation was random for group 2a subjects ($\times$), for example, Pa $\rightarrow$ Pb $\rightarrow$ G for group 2b₁ subjects ($\blacktriangle$) and, for example, Pa $\rightarrow$ G $\rightarrow$ Pb for group 2b₂ subjects ($\bullet$). These values reflect the magnitudes only of the errors, the direction depending on the displacement induced by the present or preceding prism.

becapable of storing at least two separate visuomotor correlations.

In our second condition, we tested for the possibility that humans could also learn additional correlations. Group 2 subjects were given repeated training on each of 3 conditions of prism base left, prism base right and plain glass for 10 sessions. During each session, 10 trials were given per condition (20 trials per condition were given for the first 2 sessions only). The order of presentation of each of the conditions was random for 12 subjects (group 2a) and consistent for 16 subjects (group 2b). In this latter condition, subjects were assigned to either the (fixed) training sequence of prism A $\rightarrow$ prism B $\rightarrow$ glass, or prism A $\rightarrow$ glass $\rightarrow$ prism B. Half the subjects were given the base right, half the base left prism as their first prism during the first training session.

Although each training session was also separated by 3 d, Fig. 2 shows significant inter-problem improvement for both prism conditions. Because the errors induced by the 2 prisms are in opposite directions, the error values reflect absolute magnitudes only and thus permit comparisons of the relative accuracy achieved under all 3 conditions. As in group 1, error was small during the terminal sessions. An analysis of variance of the errors for all sessions shows that the differences found between errors committed on session 1 as compared to session 10 are significant for both prisms (1st prism: $P < 0.01$; 2nd prism: $P < 0.01$). Trend analysis on the data revealed significant linear ($P < 0.001$) and quadratic components ($P < 0.001$), which reflect the overall improvement in adaptation to both prisms recorded which is more dramatic in the earlier sessions. In short, the results from group 2 show successful multistate adaptation which indicates that the human can store multiple, if conflicting, visuomotor correlations. There is little doubt, furthermore, that these multistate adaptations are learnt, as

errors are progressively eliminated and the error pattern is fully consistent with those observed in several well known 'learning to learn' paradigms not involving prisms but using a similar design^{4,5}.

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Desensitisation of muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells

THE loss of sensitivity of a tissue to an agonist (perhaps a neurotransmitter) after prolonged exposure to this compound is called desensitisation and may be specific¹ or nonspecific². Although many different mechanisms may be involved in non-specific desensitisation³, specific desensitisation (tachyphylaxis) has long been considered to involve the agonist–receptor complex^{4–7}. Our interest in the interaction of certain psychotropic drugs with muscarinic acetylcholine receptors of cultured nerve cells^{8,9} has led us to study the desensitisation of these receptors. They mediate the formation of guanosine 3',5'-cyclic phosphate (cyclic GMP)^{8–10}, a response which has yielded much information about the interactions of agonists and antagonists with the muscarinic receptor^{8,9}, although a direct coupling of this receptor and guanylate cyclase has not been demonstrated. We report here our use of this receptor effect to characterise the specific desensitisation of muscarinic acetylcholine receptors of intact mouse neuroblastoma cells (clone N1E-115).

The time course of desensitisation was very rapid with 1 mM carbamylcholine (Fig. 1a). After an apparent lag period, the rate of desensitisation appeared to be first order, with a half time ($t_{1/2}$) of about four minutes (including the lag period). Desensitisation was readily reversible, but the recovery process

followed a time course slower than that for desensitisation and apparently was not first order (Fig. 1b). A return to 100% of control values was never seen in our experiments.

The specificity of this desensitisation was determined by first incubating cells in carbamylcholine or histamine and then stimulating the thoroughly washed cells with one or the other agonist. The data (Table 1) clearly show that previous incubation of clone N1E-115 cells with carbamylcholine rendered the cells insensitive to this agonist, whereas the effects of histamine were only slightly reduced. After initial incubation, the response to histamine was markedly reduced, whereas the response to carbamylcholine was only slightly reduced (Table 1). These results suggest several points. First, the ability of carbamylcholine to decrease muscarinic receptor-mediated cyclic GMP formation is largely a specific desensitisation, as this agonist had only a slight effect on cyclic GMP formation mediated by histamine. Second, cyclic GMP formation by these cells is also mediated by histamine (by activation of H_1 receptors, E.R. submitted for publication). Third, there is a desensitisation of the histamine response which is also largely specific. Finally, these data rule out the possibility that the desensitisation is an artefact of our assay technique (for example, depletion of stores of [³H]GTP).

To determine whether there was an altered affinity of the muscarinic receptor for carbamylcholine in its desensitised state, dose–response curves were determined for cells preincubated with agonist (0.1 mM) for 3 or 6 min (Fig. 2). Carbamylcholine preincubation caused a time-dependent shift of the dose–response curve to the right and a reduction in the

Table 2 Muscarinic receptor desensitisation caused by cholinergic agonists

Agonists (0.1 mM) present during first incubation (5 min)	Carbamylcholine-stimulated [³ H]cyclic GMP formation d.p.m. (per 10 ⁶ cells ± s.e.m.)	% control
None	35,700 (±1,200)	—
Acetylcholine*	4,500 (±800)	13†
Acetyl-β-methylcholine*	10,900 (±600)	31†
Carbamylcholine	16,300 (±900)	46†
Oxotremorine	21,800 (±500)	61†
Bethanechol	28,500 (±1,700)	80†

Mouse neuroblastoma clone N1E-115 cells (subculture 19) were preincubated without agonists (control) or with the indicated agonists (0.1 mM) for 5 min, washed three times and then assayed in triplicate for [³H]cyclic GMP formation stimulated by carbamylcholine (1 mM) as described in the legend to Fig. 1. The data are the increases over basal levels of radioactivity, and averaged 1,900 d.p.m. per 10⁶ cells (range: 1,650–2,200). There were approximately 2.8×10^5 cells per assay.

*In the presence of 0.1 mM physostigmine (added 15 min before agonist).

† $P < 0.0005$ relative to control.

‡ $P < 0.0125$ relative to control.

Table 1 Specificity of carbamylcholine desensitisation

Agonist present during first incubation period (30 min)	Agonist present during second incubation period (30 s)	Agonist-stimulated [³ H]cyclic GMP formation (d.p.m. per 10 ⁶ cells ± s.e.m.)	% control
None	Carbamylcholine	46,500 (±2,400)	—
None	Histamine	56,400 (±1,000)	—
Carbamylcholine	Carbamylcholine	6,900 (±500)	15*
Carbamylcholine	Histamine	46,000 (±4,200)	82†
Histamine	Carbamylcholine	39,300 (±2,200)	85†
Histamine	Histamine	3,100 (±500)	6*

Mouse neuroblastoma clone N1E-115 cells (subculture 19) were preincubated without agonists (control), with carbamylcholine (1 mM), or with histamine (0.1 mM) for 30 min, washed 3 times and then assayed in triplicate for [³H]cyclic GMP formation stimulated by carbamylcholine (1 mM) or histamine (0.1 mM) during a 30-s incubation period. Details of the procedures are presented in the legend to Fig. 1. The data are the increases over basal levels of radioactivity, which were, in d.p.m. per 10⁶ cells, 13,000, 19,000 and 15,000 for control, carbamylcholine and histamine preincubated cells, respectively. There were approximately 2.4×10^5 cells and 1 mg protein per assay.

* $P < 0.0005$ relative to respective control.

† $P < 0.05$ relative to respective control.

maximum response. The concentration of agonist causing half-maximal cyclic GMP formation was ≈ 0.10 mM for control, and ≈ 0.4 and 0.5 mM for cells preincubated for 3 and 6 min, respectively. One possible interpretation of these data is that specific desensitisation of the muscarinic receptor of these cells reflects a decreased affinity of the receptor (assuming no 'spare' receptors) and a decreased number of binding sites for agonists. Using a radioactively labelled muscarinic antagonist in binding studies with smooth muscle, Young also obtained data which suggest that desensitisation of muscarinic receptors is associated with a decreased affinity for carbamylcholine¹¹. It was further suggested that in binding studies, the Hill coefficients (n_H) of < 1 were a result of this desensitisation. Hill coefficients for all the dose–response curves of Fig. 2 were ≈ 1.5 , while in a series of control dose–response curves, n_H values have ranged over 1–1.6. Therefore, our data and those of Young may explain why

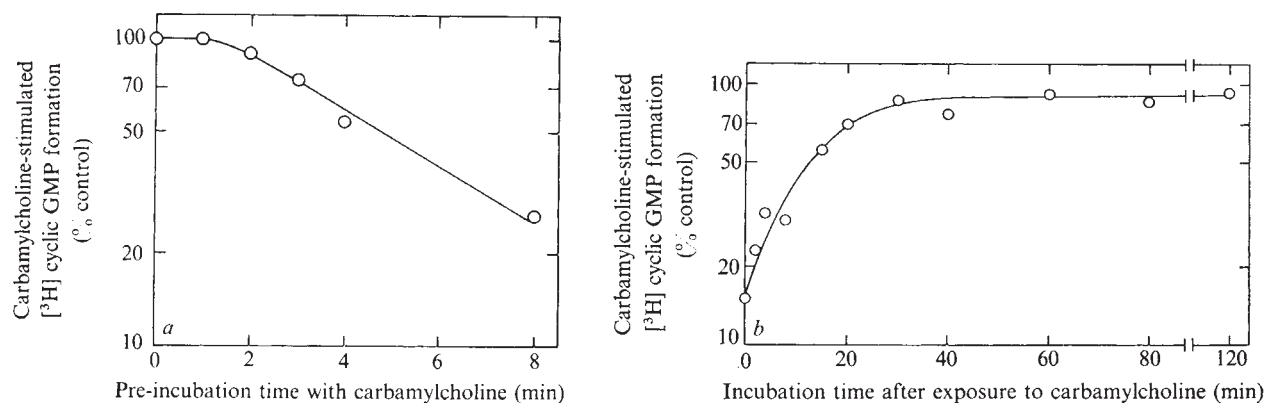


Fig. 1 Time course for desensitisation (a) of muscarinic acetylcholine receptor effects of cultured nerve cells and for reversal of this desensitisation (b). Mouse neuroblastoma clone NIE-115 cells (subcultures 16–18) were cultured as previously described^{8,9}. Before assay, cells were maintained in stationary phase (requiring daily medium change) for at least 1 week. Subculture of cells was achieved by incubation in a modified Puck's D₁ solution (medium I) (ref. 13). There was no evidence of mycoplasma (PPLO) in these cells as determined in 3 tests: microbiological assay, fluorescent microscopy using Hoechst 33258 stain¹⁴, and scanning electron microscopy. To determine the time course for desensitisation, cells were prepared for the assay of carbamylcholine-stimulated [³H]cyclic GMP formation (E.R., Prendergast and Divinetz-Romero, submitted) with modifications as detailed below. Cells were washed free of growth medium and dissociated from the surface of flasks by incubation with medium I. They were collected by low-speed centrifugation (500 r.p.m. in Damon/IEC CRU-5,000 centrifuge), and re-suspended at a density of approximately 8×10^6 cells per ml in phosphate buffered sodium chloride solution (medium II) containing the following (in mM): NaCl, 110; KCl, 5.3; CaCl₂, 1.8; MgCl₂, 1.0; Na₂HPO₄, 2.0; glucose, 25; sucrose, 70 (pH 7.4, 340 m Osm l⁻¹). The cell suspension was then placed in a 25 ml Erlenmeyer flask to which was added [^{8-³H}]guanine (Amersham/Searle, 1 μ M final concentration, 1 μ Ci ml⁻¹) and rotated at 37 °C for 45 min at 80 r.p.m. (Gyratory shaker, Model G2, New Brunswick). Equal aliquots of cells were then placed in Erlenmeyer flasks and the volume of these suspensions was brought to 2 ml with medium II. At zero time, carbamylcholine chloride (Sigma, 1 mM final concentration) was added to one of the rotating flasks. At the indicated times a 300- μ l aliquot of the cell suspension was removed from each flask, placed in 2 ml of medium II at room temperature and collected by low speed centrifugation. The supernatant was discarded and cells were washed free of carbamylcholine and/or extracellular radioactivity by resuspending the cells three times in 2 ml of medium II and collecting them by centrifugation. After the last centrifugation, cells were suspended in 1 ml of medium II, distributed in triplicate (280- μ l aliquots) into wells of a multiwell tray (Disposo trays FBI6-24TC, Bellco Glass) and incubated at 37 °C at 80 oscillations per min in a shaker bath (model 25,GCA, Precision Scientific). The time from the removal of the aliquots from the two flasks to the addition of carbamylcholine to the cells in wells was approximately 10 min. Then 20 μ l of medium II with or without carbamylcholine (1 mM final concentration) was added to the cells for 30 s. After stopping the reaction by the addition of 30 μ l of ice-cold 50% (w/v) trichloroacetate, the material was processed as previously described^{8,9}. Efficiency for counting tritium using 10 ml QuantafloorTM (Mallinckrodt) and a double-label program on a Searle Isocap/300 scintillation counter was 35%. The calculated d.p.m. for each sample incorporated corrections for quench (using external standard ratio), for c.p.m. of [^{8-¹⁴C}]cyclic GMP (internal standard) in the tritium channel (efficiency $\approx$ 20%), and for recovery of [^{8-¹⁴C}]cyclic GMP ($\approx$ 85%). To determine the time course for the reversal of desensitisation (b), cells were processed as described above except that one group of cells was exposed to carbamylcholine (1 mM final concentration) for 30 min. Control and carbamylcholine-treated cells were then washed and distributed into wells in triplicate. Zero time began with the addition of cells to wells. Total radioactivity from cells and basal radioactivity from the cation-exchange column were similar for control cells and for cells exposed to carbamylcholine for prolonged periods. Data in (a) and (b) are averages of three independent experiments.

in binding assays, agonists are less effective in displacing antagonists than would be expected from biological data. However, the reason for an $n_H < 1$ for agonists in binding studies remains unclear.

The apparent potency of several cholinergic agonists in effecting desensitisation was determined by first incubating cells for 5 min with 0.1 mM agonist and then stimulating the thoroughly washed cells for 30 s with carbamylcholine (1 mM). The results (Table 2) gave the following rank order of potency for desensitisation: acetylcholine > acetyl- β -methylcholine > carbamylcholine > oxotremorine > bethanechol. As with other receptor systems, this rank order of potency correlates well with the potency of these agonists in stimulating the muscarinic receptor in the cells (E.R., unpublished data). Also, atropine, a muscarinic receptor antagonist, can prevent most of the desensitisation caused by carbamylcholine. Thus, cells incubated for 10 min with atropine (10^{-8} M), followed by 4 min with carbamylcholine (0.5 mM) plus atropine (10^{-8} M), and then washed free of these drugs, showed a 15% loss of sensitivity to carbamylcholine. Cells preincubated with carbamylcholine alone showed a 60% loss of sensitivity, while preincubation with atropine alone had no effect on carbamylcholine-stimulated cyclic GMP formation by the washed cells.

Studies on the effects of temperature showed that desensitisation was markedly reduced at 22 °C and did not occur at 4 °C

(data not shown). In addition, carbamylcholine-stimulated cyclic GMP formation does not occur at 22 °C. The reversal of desensitisation was also markedly slowed by reduced temperatures.

Removal of Na⁺, K⁺, Mg²⁺ or Ca²⁺ from the incubation medium had no effect on desensitisation by carbamylcholine (data not shown). Also, the muscarinic receptor-mediated cyclic GMP response in these cells is virtually dependent on external calcium ions (E.R., Prendergast and Divinetz-Romero, submitted), suggesting that cyclic GMP is not involved in receptor desensitisation.

In conclusion, the specific desensitisation of the muscarinic acetylcholine receptor of cultured nerve cells, like that of other receptor systems, is a time-dependent process that is reversible; it is effected by agonists with an apparent potency which reflects their potency in causing receptor stimulation; it is blocked by a specific antagonist and is temperature dependent. However, unlike the desensitisation of the nicotinic acetylcholine receptor⁵ or the β -adrenergic receptor⁶, this desensitisation may be associated with a decreased affinity of the muscarinic receptor for carbamylcholine and a decreased number of binding sites for this agonist. Finally, because we have been unable to demonstrate alterations in binding of the potent muscarinic antagonist [³H]quinuclidinyl benzilate¹² to these cells under conditions which caused desensitisation of the cyclic GMP

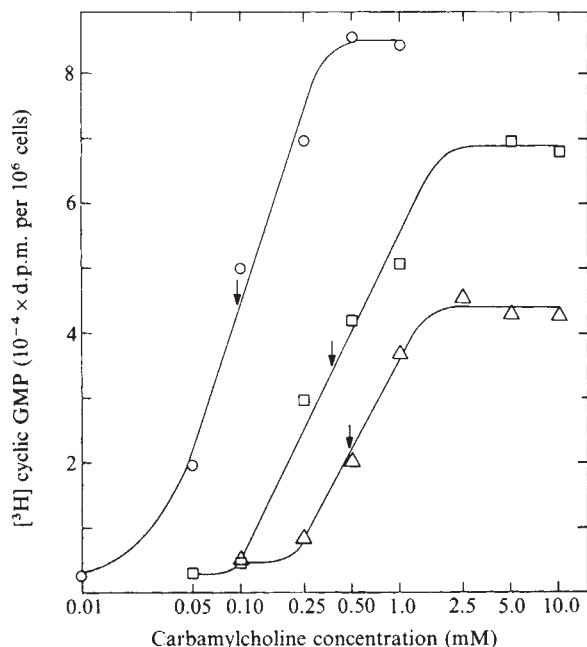


Fig. 2 Effect of preincubation of cultured nerve cells with carbamylcholine on the carbamylcholine dose-response curve. Mouse neuroblastoma clone NIE-115 cells (subculture 19) were preincubated with carbamylcholine (0.1 mM final concentration), washed three times and then assayed in duplicate for carbamylcholine-stimulated [3 H]cyclic GMP formation at the indicated concentrations of carbamylcholine, as described in the legend to Fig. 1. The data are increases over basal levels of radioactivity, and were, in d.p.m. per 10^6 cells, 4,200, 5,200 and 5,100 for control, and 3- and 6-min carbamylcholine-treated cells, respectively. Arrows indicate the concentration of carbamylcholine at half-maximal stimulation. There were approximately 2.6×10^8 cells per well, as determined by an electronic cell counter (Model ZF, Coulter Electronics) and 690 μ g protein, as determined by a modification of the method of Lowry *et al.*¹⁵, with bovine serum albumin as a standard. $\circ$, control; $\square$, carbamylcholine preincubated cells, 3 min; $\triangle$, carbamylcholine preincubated cells, 6 min.

response (E.R., unpublished data), the antagonist binding site may not be involved in this desensitisation.

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LSD labels a novel dopamine receptor in molluscan nervous system

ELECTROPHYSIOLOGICAL studies on molluscan ganglia have provided evidence for the presence of two pharmacologically distinct dopamine responses—a hyperpolarising response which is inhibited by low concentrations of ergot alkaloids and related drugs, and a depolarising response which is sensitive to neuroleptics such as haloperidol¹⁻³. Although dopamine receptors in the mammalian central nervous system (CNS) have been detected directly by the binding of a number of 3 H-labelled ligands, including the lysergic acid derivatives lysergic acid diethylamide (LSD) and dihydroergocryptine⁴⁻⁸, no such studies have been reported with molluscan nervous system. We report here that 3 H-LSD binds to both dopamine and serotonin (5-HT) receptors in molluscan ganglia, but that conditions can be obtained which allow the two receptors to be studied separately. This is the first demonstration that dopamine and 5-HT receptors may be investigated individually in the same tissue using a single ligand. The pharmacological characteristics of the dopamine-sensitive 3 H-LSD binding are compatible with the properties of the dopamine receptor which mediates hyperpolarisation in certain molluscan neurones. These receptors are, however, different from the dopamine receptors previously described in the mammalian CNS.

Binding studies were carried out on a particulate fraction derived from the circumoesophageal ring of ganglia, which comprises the CNS of the Roman snail *Helix pomatia*. Specific

Table 1 Effect of putative neurotransmitters on specific [3 H]-LSD binding to snail ganglia particulate fraction

Experiment	Agent	Concentration	Inhibition of specific [3 H]-LSD binding (%)
1	5-HT	10 μ M	32
	Dopamine	10 μ M	35
	Noradrenaline	10 μ M	0
	Octopamine	10 μ M	0
	Histamine	10 μ M	0
2	5-HT	3 μ M	29
	Dopamine	3 μ M	32
	5-HT + dopamine	3 μ M	67 (61)
	5-HT	10 μ M	39
	Dopamine	10 μ M	57
	5-HT + dopamine	10 μ M	100 (96)
	5-HT	30 μ M	58
	Dopamine	30 μ M	64
	5-HT + dopamine	30 μ M	100 (100)
	5-HT	500 μ M	100
	Dopamine	500 μ M	93
	5-HT + dopamine	500 μ M	100 (100)

Helix pomatia were decapitated and the circumoesophageal ring of ganglia dissected. Ganglia were homogenised (60 mg wet weight per ml) in cold buffer (0.2 M sucrose, 2 mM Tris-HCl, pH 7.3) using a Polytron (30 s; setting 5). The homogenate was centrifuged at 750g for 15 min at 4 °C and the supernatant decanted. The pellet was resuspended in the same volume of cold buffer and recentrifuged. The combined supernatants were centrifuged at 4 °C for 30 min at 15,000g, and the resulting pellet washed once in 2 mM Tris-HCl, pH 7.3. The pellet was finally resuspended in this solution to a protein concentration of 1-2 mg per ml and stored at -70 °C until use. Control experiments demonstrated that there was no difference between fresh and pre-frozen membranes in terms of the amount of specific or nonspecific [3 H]-LSD binding observed, and that the ability of 5-HT or dopamine to compete with this binding was unaltered. The binding reaction mixture contained membrane protein (20-100 μ g per tube), 50 mM Tris-HCl, pH 7.3, 1.8 mM ascorbic acid, [3 H]-d-LSD (specific activity 11.1 Ci mmol⁻¹), and various drugs as indicated in a volume of 500 μ l. In experiments 1 and 2 the [3 H]-LSD concentrations were 1.0 nM and 0.2 nM, respectively. After 10 min at 37 °C, the reaction was terminated by rapidly adding 5 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.3) and filtering immediately through a Whatman GFB glass fibre filter mounted in a suction apparatus. The filter was then washed 4 times with 5 ml volumes of the same buffer. The time taken for filtering and washing was 5-10 s. Radioactivity bound to trapped particles on the filter was counted in a liquid scintillation counter using a Triton X-100-toluene-based scintillation fluid. Values in parentheses are those expected theoretically from an additive effect of 5-HT and dopamine. Specific [3 H]-LSD binding in the absence of neurotransmitters was 298 ± 13 c.p.m. in experiment 1 (6% of added label) and 213 ± 8 c.p.m. in experiment 2 (18.4% of added label).

binding of ^3H -LSD was defined as the difference between the radioactivity bound in the absence and presence of $10\ \mu\text{M}$ unlabelled *d*-LSD. The binding remaining in the presence of this concentration of *d*-LSD ('nonspecific') was linearly related to the amount of radioactivity added, and consisted largely of binding to the glass fibre filter. Using ^3H -LSD concentrations below $2.0\ \text{nM}$, nonspecific binding was less than 10% of specific binding; it rose to 30% with $8.0\ \text{nM}$ ^3H -LSD. The conditions used for the measurement of specific binding were optimal with respect to time of incubation, pH and ionic conditions; these data will be described in detail elsewhere (A. H. D., F. B. and I. B. L., in preparation).

In initial studies using a ^3H -LSD concentration of $1\ \text{nM}$, various putative neurotransmitters were tested for their ability to decrease specific ^3H -LSD binding. At concentrations of $10\ \mu\text{M}$ or less, only dopamine and 5-HT were effective inhibitors; noradrenaline, octopamine and histamine were inactive (Table 1). Moreover, dopamine and 5-HT were probably displacing ^3H -LSD from different receptors, as, at concentrations below $100\ \mu\text{M}$, their effects were additive (Table 1). With higher neurotransmitter concentrations ($\geq 500\ \mu\text{M}$), binding was essentially abolished by either agent acting alone, suggesting that the two sites exhibit specificity for 5-HT or dopamine only at lower transmitter concentrations. Subsequent experiments were directed towards finding conditions in which ^3H -LSD labelled only the dopamine-sensitive site (D site) or the 5-HT-sensitive site (S site). We attempted to discriminate between the D and S sites by finding, for example, a concentration of 5-HT which would largely abolish binding of ^3H -LSD to the S-site, without affecting binding to the D-site. Preliminary experiments indicated that a concentration of 10^{-4}M 5-HT would be appropriate. As shown in Fig. 1*a*, the ^3H -LSD binding displaced by this concentration of 5-HT was that which was insensitive to dopamine. Comparable

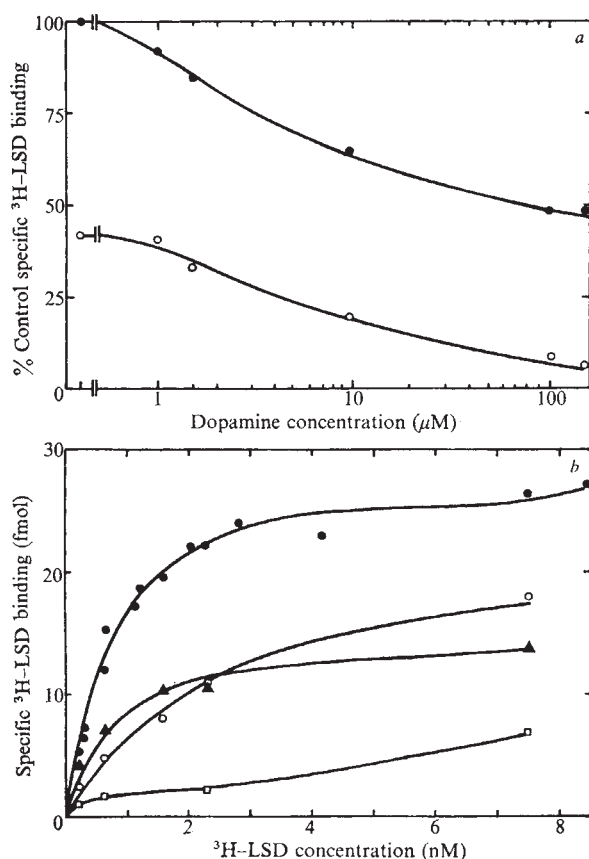
results were obtained when the experiment was reversed, that is, 10^{-4}M dopamine displaced that portion of ^3H -LSD binding which was insensitive to 5-HT (data not shown).

The dependence of the ^3H -LSD binding on LSD concentration is shown in Fig. 1*b*. Total specific binding was saturable with a K_D of $1.0\ \text{nM}$ (determined by Scatchard analysis, not shown). The K_D for ^3H -LSD binding to the D-site (measured in the presence of 10^{-4}M 5-HT) was $0.5\ \text{nM}$, and for the S-site (measured in the presence of 10^{-4}M dopamine) $1.2\ \text{nM}$. When both agents were present simultaneously, the resultant inhibition of ^3H -LSD binding was that expected on an additive basis (Fig. 1*b*), confirming that these neurotransmitter concentrations were appropriate for dissecting the two sites. Figure 1*b* also reveals that there are approximately equal numbers of D and S sites in this preparation.

Table 2 Effect of various agents on dopamine-sensitive [^3H]-LSD binding

Agent	Inhibition of dopamine-sensitive [^3H]-LSD binding $K_i\ (\mu\text{M})$
Dopamine and related compounds	
Epinine	2.7
Dopamine	4.0
DPI	14.0
L-noradrenaline	54.0
L-isoproterenol	100.0
β -phenylethylamine	> 200.0
5-HT and related compounds	
N-methyltryptamine	0.34
Bufotenine	2.0
6,7-dihydroxytryptamine	4.0
Tryptamine	13.0
6-hydroxytryptamine	22.0
5-methoxytryptamine	50.0
N-methyl 5-hydroxytryptamine	60.0
5,6-dihydroxytryptamine	100.0
5-hydroxytryptamine	> 200.0
Ergot alkaloids and derivatives	
<i>d</i> -LSD	0.0005
<i>l</i> -LSD	10.0
Ergotamine	0.0003
Ergometrine	0.0008
Dihydroergocryptine	0.010
Methysergide	0.010
Neuroleptics	
<i>d</i> -butaclamol	0.0046
<i>l</i> -butaclamol	54.0
Chlorpromazine	0.16
<i>cis</i> -chlorprothixene	0.20
<i>trans</i> -chlorprothixene	0.46
Spiroperidol	0.34
Haloperidol	0.38
<i>cis</i> -flupenthixol	10.0
<i>trans</i> -flupenthixol	6.0
Pipamperone	20.0

Fig. 1 Inhibition of specific [^3H]-LSD binding to *Helix* particulate fraction by dopamine and 5-HT. *a*, Effect of different concentrations of dopamine on the binding of $2\ \text{nM}$ [^3H]-LSD in the absence (●) or presence (○) of $100\ \mu\text{M}$ 5-HT. *b*, Saturation of specific [^3H]-LSD binding: ●, control; ▲, plus $100\ \mu\text{M}$ 5-HT; ○, plus $100\ \mu\text{M}$ dopamine; □, plus $100\ \mu\text{M}$ 5-HT and $100\ \mu\text{M}$ dopamine.



Helix ganglia particulate fractions were assayed with 4–8 concentrations of drug in duplicate or triplicate. All tubes contained $100\ \mu\text{M}$ 5-HT to restrict [^3H]-LSD binding to the dopamine site. The concentrations of drugs required to inhibit binding by 50% (IC_{50}) were calculated and converted to K_i values according to the equation $K_i = \text{IC}_{50} (1 + c/K_D)$, where c is the concentration of radioactive ligand ($2\ \text{nM}$) and K_D is its dissociation constant (taken as $0.5\ \text{nM}$ for dopamine-sensitive [^3H]-LSD binding).

The results of a preliminary pharmacological investigation of the dopamine receptor (D-site) are summarised in Table 2. Epinine (*N*-methyldopamine) and dopamine were the most potent of the catecholamines tested as competitors of dopamine-sensitive ^3H -LSD binding (Table 2). *l*-Noradrenaline and *l*-isoproterenol were respectively 20 and 37 times less active than epinine. This order of potency is similar to that seen for both inhibitory and excitatory physiological responses to dopamine in molluscs^{3,9} (although isoproterenol is generally inactive as a dopaminergic agonist on the inhibitory dopamine receptor, and 300 times less active than dopamine on the excitatory receptor³). Furthermore, (3,4-dihydroxyphenylamino)-2-imidazoline (DPI)

was a potent inhibitor of dopamine-sensitive ^3H -LSD binding (Table 2), and electrophysiological studies have shown it to be a potent dopaminergic agonist at hyperpolarising (but not depolarising) sites¹⁰. Several tryptamine derivatives (with the exception of most 5-substituted compounds) were effective inhibitors of binding (Table 2). This is consistent with the report that tryptamine and 6-hydroxytryptamine exert a weak dopamine-like hyperpolarising action on certain *Helix* neurones¹¹.

These data favour the hypothesis that ^3H -LSD labels the dopamine receptor which mediates hyperpolarisation in these cells. With this in mind, two main groups of psychoactive agents were tested for competition with ^3H -LSD binding. Ergot alkaloids and derivatives such as ergotamine, ergometrine and *d*-LSD, which are potent inhibitors of dopamine-induced hyperpolarising responses in invertebrates^{3,12,13} (and are much less active against depolarising responses), were very potent inhibitors of dopamine-sensitive ^3H -LSD binding (Table 2). A number of neuroleptics which, conversely, are potent blockers at depolarising dopamine synapses but are relatively inactive at hyperpolarising dopamine sites^{3,14}, were much less effective than the ergot drugs in displacing dopamine-sensitive [^3H]-LSD binding. The neuroleptics are more potent dopamine receptor blockers in vertebrates^{4,5} than in *Helix* (Table 2), and the order of potency reported for these agents in calf brain does not parallel that found in molluscan ganglia. In addition, although both *d*-butaclamol and the *cis*-thioxanthene drugs, flupenthixol and chlorprothixene, are stereospecific inhibitors of binding at [^3H]-haloperidol-labelled vertebrate dopamine receptors^{4,5,15}, only *d*-butaclamol was a stereospecific inhibitor of [^3H]-LSD binding in snail brain (Table 2). While the spectrum of drug action on these [^3H]-LSD-labelled dopamine receptors is clearly different from that previously described in vertebrate tissues using neuroleptic ligands, a recent report⁷ suggests that approximately 15–20% of specific [^3H]-LSD binding to calf caudate membranes involves dopamine receptors. If a pharmacological characterisation of these receptors yields data similar to those reported here, it would be consistent with behavioural experiments¹⁶ which suggest¹⁷ that the vertebrate CNS also contains ergometrine-sensitive dopamine receptors analogous to those in the molluscan CNS.

The results presented above are compatible with the theory that [^3H]-LSD labels the dopamine receptor which mediates a hyperpolarising response in molluscan neurones. Electrophysiological studies, based on the pharmacology reported here, are now underway to confirm this hypothesis.

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Tentative identification of β -adrenoreceptor subunits

MANY hormones and neurotransmitters interact with specific receptors which are coupled to the enzyme adenylate cyclase^{1–4}. Sutherland *et al.* were the first to suggest that the receptor constitutes the regulatory subunit possessing the allosteric effector site of the adenylate cyclase enzyme. Recently, two lines of experimental evidence have supported the postulate that the β -adrenoreceptor and the membrane bound enzyme adenylate

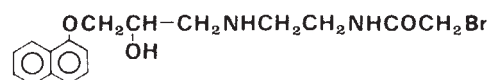


Fig. 1 Structure of the β -adrenoreceptor affinity label NHNP-NBE. The synthesis of *N*-[2-hydroxy-3-(1-naphthoxy)-propyl]-*N'*-bromoacetyl-ethylenediamine (NHNP-NBE) was described earlier^{10,11}. The compound was shown to inhibit irreversibly the catecholamine-dependent adenylate cyclase but not the NaF-dependent adenylate cyclase activity of turkey erythrocytes^{9,10}. The affinity label irreversibly blocks the specific binding of [^3H]-propranolol^{9,10} and of [^{125}I]-hydroxybenzylpindolol¹⁹.

cyclase are situated on separate macromolecules. Lefkowitz *et al.*^{5,6} succeeded in separating the β -adrenoreceptor from the adenylate cyclase by molecular sieve chromatography of digitonin solubilised from erythrocyte membranes. Schramm

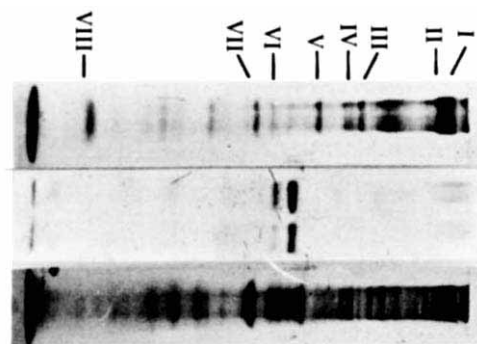


Fig. 2 The identity and the molecular weight of the β -adrenoreceptor submit. Rat skeletal myoblasts (L6P cells) were grown on a collagen matrix as described earlier^{12,13}. After removal of the growth medium, the cells were washed with 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl. Then the cells were incubated for 10 min at 37 °C with 5.0×10^{-6} M of the tritiated affinity label [^3H]-NHNP-NBE (5.5 Ci mmol^{-1}) in the presence or absence of 1.0×10^{-6} M hydroxybenzylpindolol (HYP). Each growth bottle was then washed 20 times with 8.0 ml of 0.02 M Tris-HCl, pH 7.4, containing 0.13 M NaCl. The cells were removed from the growth bottles by scraping with a rubber 'policeman' and centrifuged at 400g for 5 min. They were resuspended in the same buffer and recentrifuged. The washing procedure was repeated twice. Protein was determined by the Lowry method¹³. The cells were dissolved in the electrophoresis buffer which contained 10% sodium dodecyl sulphate (SDS)¹⁵, and identical amounts of protein (100 μg) were applied for slab gel electrophoresis using the set up of Fairbanks *et al.*¹⁵. The slab gels were stained with Coomassie blue, photographed, and then subjected to autoradiography for 8 weeks according to the procedure developed by Bonner and Lasky¹⁶. The molecular weight of the labelled bands was determined by comparing their mobilities with those of proteins from human erythrocyte membranes with known molecular weights. The latter were subjected to electrophoresis in the presence of SDS in parallel to the labelled L6P cells. The molecular weight of the proteins from the human erythrocytes are as follows¹⁷: Band I, 240,000; II, 215,000; III, 89,000; IV, 72,000; V, 43,000; VI, 35,000; VII, 29,000. The two protein bands from L6P cells labelled with the affinity label have an apparent molecular weight of 37,000 and 41,000. *a*, Human erythrocyte membranes; *b*, L6P cells labelled with the [^3H]-affinity label in the absence of HYP; *c*, L6P cells labelled with the [^3H]-affinity label in the presence of HYP; *d*, protein stain (Coomassie blue) of L6P proteins.

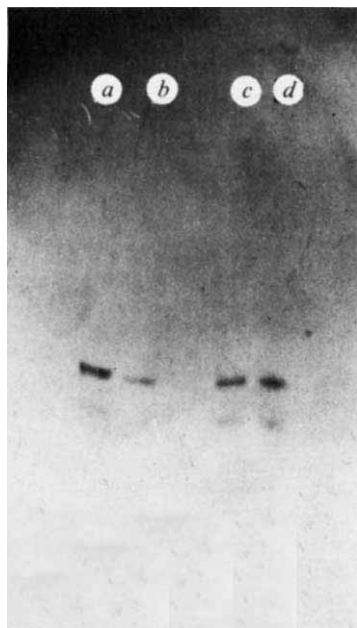


Fig. 3 The affinity labelling of turkey erythrocyte membranes. Fresh turkey blood was washed 3 times with 0.15 M NaCl. The lymphocyte-free packed erythrocytes were diluted with isotonic buffer (0.02 M Tris-HCl, pH 7.4, 0.13 M NaCl) and the [3 H]-affinity label was added to a final concentration of 5.0×10^{-6} M. This mixture was incubated at 37 °C for 5 min with occasional stirring. In a parallel experiment the treatment with the affinity label was repeated in the presence of 2.5×10^{-5} M HYP. The cells were then centrifuged at 400g for 5 min, resuspended in isotonic buffer, and centrifuged. The washing procedure was repeated 4 times. The cells were then resuspended in hypotonic buffer and broken using a Dounce homogeniser (40 strokes using a tight fit Dounce homogeniser). The broken cells were diluted 1:2 in 0.03 M sodium phosphate buffer, pH 7.4, containing 2 mM MgCl_2 and 1 mM EDTA, and the sample (4.0 ml) was applied on a discontinuous sucrose gradient: 10 ml 40% sucrose and 5.0 ml 25% sucrose made in the above buffer. The gradient was centrifuged at 19,000g for 40 min. The membranes were collected from the 25–40% interface and washed in 0.03 M sodium phosphate buffer. Then they were dissolved according to Fairbanks *et al.*¹⁵. 100 μ g membrane protein were applied for slab gel electrophoresis. For comparison, L6P cells labelled with affinity label were run in parallel. The electrophoresis was allowed to proceed for a longer time in order to resolve the two protein bands and to compare their mobility with the labelled bands from L6P cells. *a*, L6P cells labelled with [3 H]-affinity label in the absence of HYP; *b*, L6P cells labelled with [3 H]-affinity label in the presence of HYP; *c*, turkey erythrocyte membranes labelled with [3 H]-affinity label in the presence of HYP; *d*, turkey erythrocyte membranes labelled with [3 H]-affinity label in the absence of HYP.

et al.^{7,8} have shown that the β -adrenoreceptor can be transferred from turkey erythrocyte cells and become coupled to adenylate cyclase of Friend erythroleukaemic cells by virus induced cell fusion. Using a tritium-labelled affinity label for the β -adrenoreceptor^{9–11}, we report here the tentative identification of the β -adrenoreceptor subunits from rat skeletal myoblasts grown in culture (L6P cells) and from turkey erythrocytes.

The compound *N*-[2-hydroxy-3-(1-naphthylthio)-propyl]-ethylenediamine (NHNP-E) (refs 9–11) was tritiated by Radiochemical Centre, Amersham, to a specific radioactivity of 5.5 Ci mmol⁻¹. This compound is a potent β -adrenergic blocker, as measured both by inhibition of *l*-adrenaline-dependent adenylate cyclase and by inhibition of [3 H]-propranolol binding to turkey erythrocyte membranes^{9–11}. The NHNP-E β -receptor dissociation constant, as measured by these two techniques, was found to be $2.0(\pm 1.0) \times 10^{-7}$ M. The compound was then reacted with bromoacetic acid *N*-hydroxy succinimide ester, as previously described^{10,11}, to yield the specific β -adrenoreceptor affinity label *N*-[2-hydroxy-3-(1-naphthylthio) - propyl] - *N'* - bromoacetyl - ethylenediamine

(NHNP-NBE) (Fig. 1). NHNP-NBE inhibits irreversibly *l*-adrenaline-dependent adenylate cyclase activity, but has no effect on the NaF stimulated activity^{9–11}. The affinity label was also shown to block irreversibly the binding of [3 H]-propranolol^{9–11} and of [125 I]-hydroxybenzylpindolol to the β -receptor sites¹⁹. The tritium-labelled affinity label was then reacted with intact L6P cells or with intact turkey erythrocytes using experimental conditions previously developed^{9,10} and described in the legends to Figs 2 and 3. From the autoradiogram of Figs 2 and 3, it is apparent that only two proteins are labelled on treatment of the L6P cells or of turkey erythrocytes with the tritiated affinity label. Hydroxybenzylpindolol (HYP), a potent β -adrenergic blocker¹⁸, when applied simultaneously with the [3 H]-affinity label, partially protects against the labelling of the two protein bands. As we have reported¹⁰, the incomplete protection offered by reversible β -agonists or β -antagonists might be due to the extreme reactivity of the affinity label. It can be seen that the labelling of the major band is significantly diminished in the presence of HYP, while the labelling of the minor band is almost completely eliminated when HYP is present in the reaction mixture. It is interesting that the labelling of the minor protein band is rather weak and appears only after a long exposure to the autoradiographic film.

The protein bands which become radioactively labelled with the affinity label constitute only a very small fraction of the total membrane protein and are barely detected by the protein stain (Fig. 2). The molecular weight of the labelled bands is in the range 37,000–41,000. As the molecular weight of digitonin solubilised β -adrenoreceptor is in the range 130,000–150,000 (ref. 5) or higher⁶, it seems that the receptor is oligomeric in structure. It is not clear whether the two labelled protein bands (Fig. 2) represent two genuinely different subunits of the oligomeric β -adrenoreceptor. It is noteworthy that proteins of indistinguishable electrophoretic mobility become labelled by [3 H]-NHNP-NBE in L6P cells and in turkey erythrocytes. The line L6P was established by several passages of myoblasts isolated from primary rat skeletal-muscle cell cultures prepared from newborn non-inbred Wistar rats. This finding may imply that the structure of the β -adrenoreceptor is similar in different types of cells and may have been conserved during evolution.

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High incidence of virus isolation from donor and recipient tissues associated with renal transplantation

DESPITE serological evidence of increased viral infection in organ transplant recipients¹, attempts to isolate virus from donor or recipient tissues at autopsy and biopsy have been unsuccessful²⁻⁴. The fresh donor tissues available for the studies reported here offered an unusual opportunity to study virus transmission with organ transplantation, and produced evidence of frequent viral infection in apparently healthy donors. We report here that viruses could be isolated from over 80% of both donor and recipient tissue. Previous serological reports have suggested an increased incidence of herpesviruses associated with transplantation¹⁻⁵, but the high incidence reported here for tissues of donors without any apparent herpetic lesions has important implications both with respect to the transmission of herpesviruses and their relationship to neoplasia.

The random donor kidneys used in this study had for various reasons not been used for transplantation. In instances where both kidneys had been transplanted, donor liver or spleen obtained at the time of organ recovery was substituted. Recipient kidneys had been rejected and were obtained at either nephrectomy or autopsy. All tissues were obtained in sterile conditions and stored in sterile conditions in McCoy's medium or perfusion fluid (cryoprecipitated type AB plasma) at -70°C until used for virus isolation.

A cube of tissue measuring approximately 2–3 cm was minced in sterile phosphate-buffered saline (PBS), then homogenised in a hand-operated tissue grinder. The resulting suspension was filtered through two layers of sterile surgical gauze into a centrifuge tube and subjected to 30,000g at 4°C for

were sham-inoculated with virus-free medium to serve as uninoculated controls.

All tubes were inspected twice daily for significant cytopathic effect (CPE) by comparing with all the controls. Following incubation for 72 h, the contents of all tubes, including uninoculated controls, were passed irrespective of any CPE by scraping with a sterile 1.0 ml plastic pipette and transferring the contents in 0.2-ml amounts into new Wi-38 monolayers.

The procedure was repeated using cultures of Wi-38 cells prepared in either eight-compartment chamber slides (Lab-Tek, Miles Laboratories) or single slide chambers of our own design⁶ that had been seeded three days earlier with 15,000 cells in each compartment or 500,000 cells in the single slide chamber.

After another 72 h of incubation, cultures were prepared for immunofluorescent staining. The medium was aspirated, plastic dividers removed, and the monolayers air-dried at room temperature. The slides were then immersed in -20°C acetone for 10 min. To segregate the different immunofluorescent reagents, the rubber gasket was retained or vinyl ink used to subdivide the monolayer into compartments after acetone treatment of the single slide chambers.

The immunofluorescent procedure used rabbit anti-Herpesvirus hominis Type 1 and Type 2 labelled with fluorescein isothiocyanate (FITC), and sheep anti-human cytomegalovirus labelled with FITC (Microbiological Associates). Controls consisted of compartments inoculated with the passed isolate or control preparations, and stained with non-herpes conjugates (SV40 or mumps), and other compartments inoculated with medium alone and stained with herpes or non-herpes virus conjugates. Staining was based on methods previously reported⁷. The stained and coded slides were read by someone other than the one who had done the staining.

At least one virus was isolated from 12 out of 14 donors and 14 out of 15 recipients. Herpesvirus hominis Type 1 was isolated from 1 donor and 3 recipients. Herpesvirus hominis Type 2 was isolated from 5 donors and 7 recipients. Human cytomegalovirus was isolated from 11 out of 14 donors and 12 out of 15 recipients (Table 1). Isolation of all 3 viruses from the same individual occurred in 1 donor and 1 recipient. The most frequent combination was cytomegalovirus and Herpesvirus hominis Type 2 (Table 2). Cytomegalovirus, however, was the most frequently isolated of the 3 viruses studied. Study of 13 female patients (donor and recipient) yielded 21 viruses, and 18 viruses were isolated from 16 male patients (donors and recipients) (Table 3).

In other reports, virus of any type was isolated from less than 10% of routine autopsy material^{8,9}. In renal transplant cases, the rate of isolation of Herpesvirus hominis Type 1 was generally low²⁻⁴, and it was seldom isolated from donors. Isolation of Herpesvirus hominis Types 1 and 2 was also low from individuals with unsuspected herpetic infection^{10,11}, in contrast to those with herpetic lesions¹².

In our study, the frequency of cytomegalovirus isolation from tissue of both donors and recipients agreed with the high incidence of infection found in other serological studies¹⁻⁵, but our rates of isolation of Herpesvirus hominis Types 1 and 2 from both donor and recipient tissues were unexpectedly high. Donors were essentially normal individuals who had died suddenly—six from head injuries, six from intra-cranial haemorrhage, one from brain tumour and one from anaesthesia. From the 29 donors and recipients tested, Herpesvirus hominis

Table 1 Virus isolation from tissues

Source of samples*	Organ	Viruses isolated		
		CMV	HSV1	HSV2
Donor	Kidney (10)	8	1	4
Donor	Liver (2)	2	0	1
Donor	Spleen (2)	1	0	0
Recipient†	Kidney (15)	12	3	7
Cell monolayer control (42)‡	—	0	0	0

*Each sample represents a different individual.

†Recipient samples consisted of only kidney tissue.

‡Wi-38 cell monolayer compartments inoculated with resuspended cell pellets from subpassaged uninfected Wi-38 cell monolayers and stained with the above viral conjugate.

about 16 h. The supernatant was then discarded and the pellet resuspended in 5 ml of PBS, and frozen. All tissues from one individual were processed simultaneously and then frozen before proceeding to the next individual's specimens. When thawed, resuspended pellets from 10 specimens were each inoculated into two stationary monolayer tubes of Wi-38 cells (Flow Laboratories). Each isolation passage also included four uninoculated tubes, four control tubes infected with Herpesvirus hominis Type 1 (MacIntyre strain), four infected with Herpesvirus hominis Type 2 (G strain), and four infected with cytomegalovirus (American Type Culture Collection). Other tubes

Table 2 Combination of virus recovery

	All neg.	Pos.*	CMV+ HSV ₁ + HSV ₂ +	CMV+ HSV ₁ − HSV ₂ −	CMV+ HSV ₁ + HSV ₂ −	CMV− HSV ₁ − HSV ₂ +	CMV− HSV ₁ + HSV ₂ −	CMV− HSV ₁ − HSV ₂ +
Donors (14)	2	12	1	7	0	3	0	1
Recipients (15)	1	14	1	5	1	5	1	1
Total (29)	3	26	2	12 (45%)	1	8 (30%)	1	2

*Positive for at least one of the three viruses.

Types 1 and 2 were isolated from 16.

We found no significant difference between donors and recipients in rates of virus recovery, contrary to the few reports of virus isolation from donors¹. However, a high incidence of isolation of Herpesvirus hominis Type 2 from non-lesion areas of human tissue, specifically from seminal vesicles of random individuals, was reported, the virus having been recovered in 4 out of 10 autopsies¹³, a rate of recovery similar to ours of 36% and 47%, respectively, from donors and recipients. We found little difference in Herpesvirus hominis Type 2 recovery between male or female donors or recipients. The high incidence of virus isolation from relatively normal individuals would make it difficult to evaluate the significance of viral transmission in transplantation procedures, but would support the concept of exacerbating pre-existing infection in an immunosuppressed patient, as suggested by serological studies¹⁴.

Table 3 Sex distribution of viruses recovered

Females	Donors (8)*		Recipients (5)	
	No. of isolates	%	No. of isolates	%
Herpesvirus hominis 2	3	33	3	60
Herpesvirus hominis 1	1	12	2	40
Cytomegalovirus	8	100	4	80
Males				
	Donors (6)		Recipients (10)	
	No. of isolates	%	No. of isolates	%
Herpesvirus hominis 2	2	33	4	40
Herpesvirus hominis 1	0	0	1	10
Cytomegalovirus	3	50	8	80

*No. of individuals shown in parentheses.

It should be noted, however, that our high incidence of recovery of viruses is similar to other serological findings^{2,4}, although viral antibody profiles were not obtained in our study because of sera unavailability. We attribute our high rates of virus recovery to the quantity of fresh material made available through the normal procedure of obtaining and using donor kidneys. The high percentage of cytomegalovirus isolation was not surprising. What was surprising, was the disproportionately higher incidence of Herpesvirus hominis 2 in comparison with Herpesvirus hominis 1, because published reports had indicated the reverse¹⁵. The actual isolation of viruses from such a relatively large percentage of normal donors, both male and female, would complicate any attempt to relate these viruses to neoplasia or other diseases.

In conclusion, an unusually high incidence of isolation of Herpesvirus hominis 1 and 2 as well as cytomegalovirus was obtained from both 'normal' cadaver donors and renal transplant recipients. The actual isolation of viruses from such a relatively large percentage of 'normal' individuals seems to augment the difficulty of interpreting the role of herpesviruses, with respect both to viral transmission during transplantation and their relation to neoplasia.

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Persisting oncogenic herpesvirus induced by the tumour promoter TPA

PERMISSIVE cell systems for the replication of the Epstein-Barr virus (EBV) and some of the related EBV-like agents (for example, Herpesvirus papio and herpesvirus pan) have not yet been established¹. EBV is regularly demonstrated in a small number of cells from lymphoblastoid lines of B-cell origin which have been derived from certain lymphoma patients, most frequently from Burkitt's lymphoma or patients with infectious mononucleosis, but also from healthy EBV-reactive individuals. EBV DNA usually persists in cells of these lines in multiple copies^{2–4}, but spontaneous induction of viral antigens and particle synthesis occurs in a majority of the lines at a low rate. To some extent, the percentage of induced cells seems to be cell line-specific¹, and two lines which are rather high producers of EBV are the P3HR-I line of BL origin⁵ and the marmoset B95-8 line isolated from lymphocytes transformed with EBV of infectious mononucleosis origin⁶. At any time during cultivation, 2–10% of the cells reveal viral capsid antigen synthesis as determined by indirect immunofluorescence⁷. All attempts to increase the virus yield in these lines by temperature shifts or chemical or physical inducers (IUDR, mitomycin C, X rays) have usually resulted in only barely significant enhancement of viral replication^{8–10}. We now report on a very efficient induction of EBV and other oncogenic herpesviruses by the well established cocarcinogen and tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)¹¹. In addition some TPA-

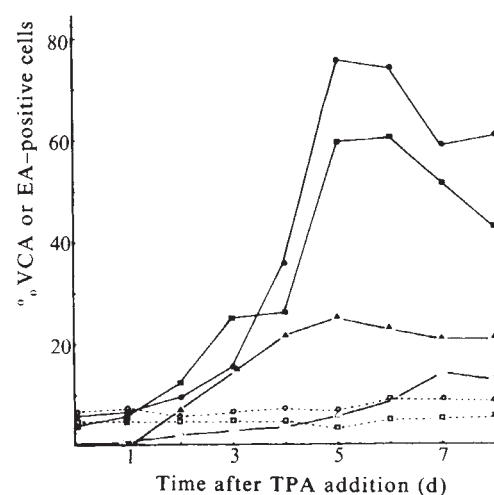


Fig. 1 Induction of EBV antigen synthesis in a P3HR-I (●), B95-8 (■) cells by TPA. 5×10^5 cells per ml were incubated with 20 ng TPA per ml. Small samples were collected every 24 h after TPA addition and assayed for the % of viral antigen-positive cells according to the procedure of Henle and Henle⁷. Staining of P3HR-I and B95-8 cells was performed with a human EA⁺ VCA⁺ serum (titre 1:320) at a dilution of 1:40. Cultures of P3HR-I (○) and B95-8 (□) without TPA treatment were analysed in parallel. The % of EA-positive cells in Raji (△) and NC 37 (▲) cells was analysed after staining with an EA⁺ VCA⁺ human serum (EA titre 1:320) at a dilution of 1:40. In the Raji control the % of EA-positive cells was below 0.01, in NC 37 it amounted to approximately 0.1%. No specific fluorescence was observed in these lines with EA⁺ VCA⁺ human sera.

treated cells show cytopathogenic changes, including polyploidisation. These observations may enable a more complete investigation of the virus-cell-gene balance hypothesis¹².

Treatment of the two producer lines P3HR-I and B95-8 and the two nonproducer lines Raji and NC37 with TPA resulted in a dramatic increase of antigen-positive cells. Viral capsid antigen (VCA) was increased in P3HR-I and B95-8, and early antigen (EA) only was induced in NC37 and Raji cells (Fig. 1). After 5 d approximately 80% of P3HR-I cells and 60% of B95-8 cells revealed characteristic immunofluorescence after indirect staining with EBV VCA-reactive sera (Fig. 2a, b). No fluorescence was observed with VCA-negative sera. After 8–10 d almost all P3HR-I cells were dead, presumably due to virus production, but some B95-8 cells survived and continued to grow. Effective virus particle production was visualised after negative staining by electron microscopy. Numerous aggregates of particles were found in every grid square after sedimentation of 5 ml of the supernatants 9 d after TPA addition, whereas only scattered individual particles were found in the controls. In the NC37 line, more than 25% of the cells were EA-positive. Maximum yields of EA-reactive Raji cells were reached 8–9 d after TPA addition.

To analyse further the induction by TPA, additional lymphoblastoid lines transformed by oncogenic herpesviruses were tested for viral antigen induction 5 d after treatment (Table 1). In every EBV-transformed line tested, some increase in EA or VCA synthesis was noted, although the frequency of viral antigen induction varied considerably. Human umbilical cord blood lymphoblasts transformed *in vitro* by EBV derived from B95-8 cells and two lines originating from patients with Hodgkin's disease belonged to the group of low responders. In animal cells the high induction rate of EBV in the B95-8 line originating from marmoset B lymphocytes transformed by EBV *in vitro*, is presented here as a point of reference. Two baboon lymphoblastoid lines harbouring herpesvirus papio showed only a moderate induction rate. In contrast, a T-cell line transformed by herpesvirus saimiri was efficiently induced after TPA treatment.

Figure 3 shows the induction rate of EA 5 d after treating Raji cells with various concentrations of TPA. The curve

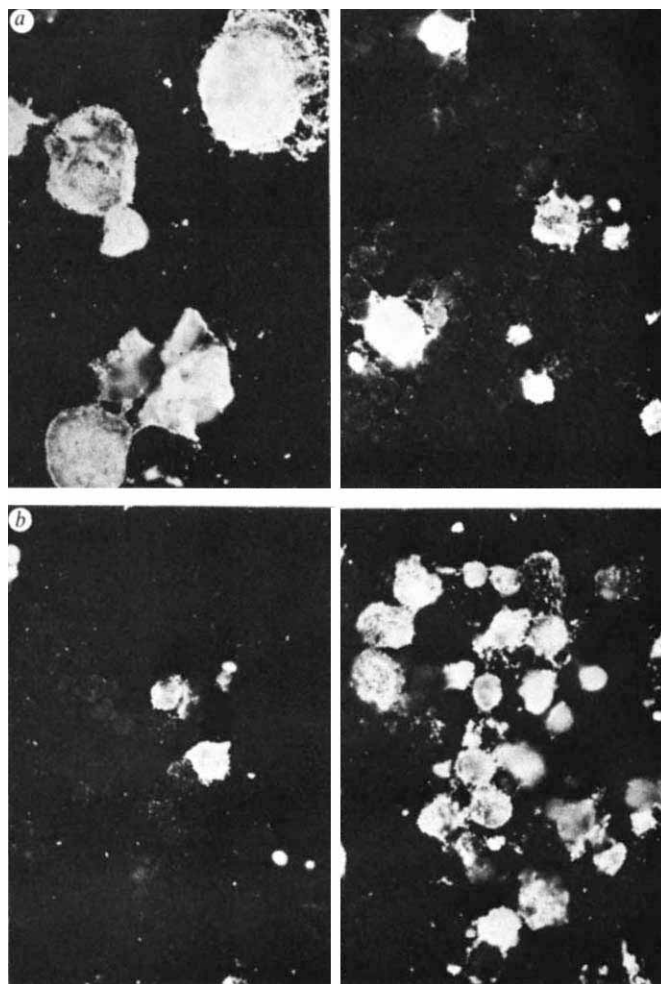


Fig. 2a, Staining for EBV-VCA in P3HR-I cells treated with TPA for 6 d (top left) and controls. The cells were treated as outlined in the legend of Fig. 1. Note the enlarged fluorescent cells in the TPA treated culture. b, Staining for EBV-VCA in B95-8 cells treated with TPA for 6 d (bottom right) and controls. B95-8 cells remain smaller after TPA induction when compared to P3HR-I.

Table 1 Induction of persisting EBV genomes in human and animal cells

Cell line	Origin	Transformed by	% Cells stained specifically for viral antigens	
			Control	5 d TPA
P3HR-I	African BL	EBV	9.1	74.3
2003	African BL	EBV	0.2	8.8
Raji	African BL	EBV	<0.01	12.2
J. I.	European BL	EBV	0.01	0.2
Ly 28	Peripheral blood NPC	EBV	0.6	3.3
QIMR-Wil	Peripheral blood leukaemia	EBV	2.8	10.0
Kaplan	I. M.	EBV	0.8	5.9
L 281	Peripheral blood H. D.	EBV	<0.01	0.5
SK 231	Peripheral blood H. D.	EBV	<0.01	0.1
NC 37	Healthy donor	EBV	0.01	13.3
α	Umbilical cord blood	EBV	<0.01	0.4
β	Lymphocytes transformed	EBV	<0.01	0.5
γ		EBV	<0.01	0.1
B 95-8		EBV	5.2	60.6
18 b	Baboon	HVP	4.7	7.7
9 b	Baboon	HVP	4.2	14.9
77/5	Marmoset	HVS	0.08	9.0

The cells were stained 5 d after addition of 20 ng per ml of TPA with a human EA⁺ VCA⁺ serum (EA titre 1:320, VCA 1:1280) at a dilution of 1:40. Staining of 18 b and 9 b cells was performed with the same serum. The HVS-transformed cells were stained with a serum (provided by B. Fleckenstein) from a HVS-tumour-bearing marmoset, at a dilution of 1:20. The lines P3HR-I, 2003 and Raji were provided by W. Henle, Philadelphia; J. I. was established by G. Bornkamm, Erlangen; Ly-28, Kaplan and NC 37 were provided by G. Klein, Stockholm, the L 281 and SK 231 lines by V. Diehl, the QIMR-Wil line by M. A. Epstein, Bristol, B95-8 by G. Miller, New Haven, the lines 18 b and 9 b, transformed by Herpesvirus papio (HVP), were provided by G. Klein, Stockholm, and the 77/5 lines, carrying genomes of herpesvirus saimiri (HVS), was established by B. Fleckenstein from a HVS-induced marmoset lymphoma¹⁴. All other EBV-transformed lines were established in this laboratory. BL, Burkitt's lymphoma; NPC, nasopharyngeal carcinoma; I. M., infectious mononucleosis; H. D., Hodgkin's disease.

suggests that the most efficient induction is achieved by approximately 20 ng per ml of TPA. Even at a concentration of 5 ng per ml a significant induction rate was noted; at higher concentrations TPA seems to be toxic. Treatment of most lymphoblastoid cells with TPA at concentrations between 5 and 160 ng per ml resulted in increased stickiness of the cells and considerable clumping. In most of the treated cultures, during the first days of treatment some of the clumps as well as individual cells attached to the glass.

Thus, TPA, one of the most active tumour promoting phorbol esters of croton oil¹, efficiently induces persisting herpesvirus genomes in lines transformed by EBV, herpesvirus saimiri and the herpesvirus papio. Preliminary data

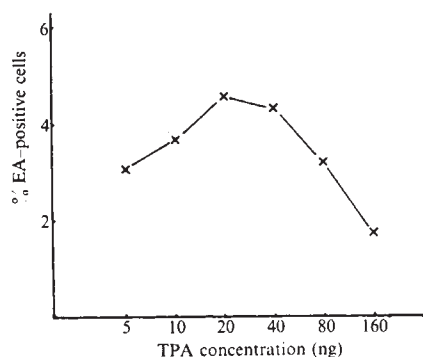


Fig. 3 Dose response of EA induction in Raji cells treated with various concentrations of TPA. 10 ml of Raji cell suspensions (5×10^6 cells per ml) were treated with TPA and analysed 5 d after treatment for EA induction as outlined in Fig. 2.

also suggest its inducing potential in cells infected by SV40 or BK-virus (H. Z. H. *et al.*, unpublished). As it is highly effective at a concentration of 3×10^{-8} M, it exceeds by several orders of magnitude the hitherto best known inducing substances like BUDR or IUdR, which are mainly effective at 7×10^{-5} M. The mechanism of virus induction by TPA is unknown. The substance stimulates DNA synthesis, as has been shown in other cellular systems¹³ and is also underlined by the high virus yields of P3HR-I cells undergoing TPA treatment. Preliminary data indicate that other tumour promoters also induce persisting viral genomes (H. Z. H. and E. H., unpublished).

The efficient induction of EBV antigens and particle synthesis in the two producer lines, P3HR-I and B95-8, seems to result in a permissive EBV system and should enable large scale molecular biological and immunological studies of this virus. The availability of a potent inducing substance of persisting viral genomes may also provide a tool for analysing the role of other viral agents in human oncogenesis and in autoimmune diseases.

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Methylation of single-stranded DNA *in vitro* introduces new restriction endonuclease cleavage sites

RESTRICTION endonucleases recognise specific sequences in DNA, and these endonucleases, especially those which generate cohesive ends, have been widely used to clone DNA¹. However, many DNAs lack sequences which are recognised by endonucleases such as *EcoRI*, *HindIII* or *BamHI*. A general method of overcoming this problem has been described recently². This approach involves the synthesis of oligonucleotides sensitive to a specific endonuclease and the blunt end ligation of these molecules to the DNA to be cloned. In contrast, we sought a method which avoids the insertion of additional nucleotides into a DNA sequence, but depends on direct modification of DNA. If a DNA sequence differs in only one base pair from the recognition sequence of a restriction endonuclease, a particular change of this base pair will generate the proper sequence. Here we describe a way of generating restriction endonuclease cleavage sites by single base changes derived after *in vitro* methylation of single-stranded DNA.

Our particular interest was the creation of unique restriction sites in the single-stranded DNA phage vector M13 mp1 (ref. 3). This phage is a derivative of the filamentous phage M13 and carries the *Escherichia coli lac* regulatory region and the information for the first 145 amino acids of β -galactosidase (EC 3.2.1.23) in a nonessential part of its genome. The synthesis of the α peptide encoded by the *lac* structural part is governed by the *lac* controlling elements; its ability to mediate α -complementation in an appropriate host can be monitored by a colour reaction on

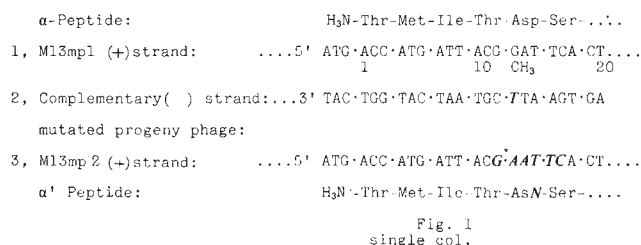


Fig. 1 Schematic representation of the single base change in the promoter proximal region of the β -galactosidase gene that leads to an *Eco*RI site. *N*-methyl-*N*-nitrosourea (gift of A. Gronenborn) was adjusted with *N*-[3 H]methyl-*N*-nitrosourea (NEN) to a final concentration of $300 \mu\text{g ml}^{-1}$ with a specific activity of 7 mCi mmol^{-1} in 98% ethanol. Single-stranded M13 mp1 DNA 1.2 mg ($100 \mu\text{g ml}^{-1}$) was reacted with 6.6 mg *N*-methyl-*N*-nitrosourea in 0.2 M sodium cacodylate buffer $\text{pH } 8.1$, 1 mM EDTA at 40°C in the dark by adding the alkylating agent in six portions to keep the initial reaction ratio of *N*-methyl-*N*-nitrosourea: base about 3:1. About 40 methyl residues were introduced per M13 mp1 molecule. This amount should produce 2 to 4 molecules of O_6 -methylguanine per phage genome (ref. 9). The reaction was stopped by adding 0.2 volumes of a mixture of 1.0 M Tris-acetate $\text{pH } 7.5$, 1.0 M β -mercapto-ethanol, 1.5 M Na-acetate, 0.05 M magnesium acetate, 1 mM EDTA. The total methylated DNA was concentrated by ethanol precipitation and used to transform CaCl_2 treated cells¹⁰. Growth was allowed for several generations and highly purified RF I molecules were isolated³.

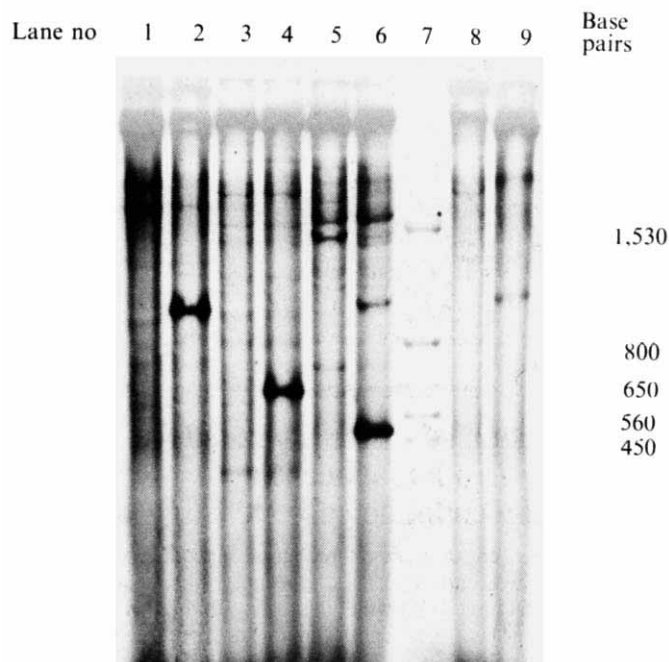


Fig. 2 Autoradiogram of a 2% agarose gel fractionation of restricted DNAs from three individual clones of M13 mp1 mutants. About 100 μ g of RF1 molecules derived after alkylation and propagation were digested by *EcoRI*. Linearised RF III molecules were enriched by separation of the reactants on 0.8% agarose gels, excised from the gels and purified by DEAE-cellulose chromatography. DNA was extracted from agarose with 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, the agarose pelleted by centrifugation, and the supernatant containing the DNA was applied to a DEAE-cellulose column (2.5 $\times$ 10 mm). The DNA was eluted in a volume of 0.2 ml of 1.0 M NaCl in the same buffer, dialysed against H₂O and subjected to gel electrophoresis or recircularised via the cohesive ends in the presence of T₄ DNA ligase. Recircularised molecules were used to transfect a sensitive host. Supercoiled DNA was prepared from individual plaque formers, and labelled with ³²P- α -dATP (NEN) by nick translation¹¹. After digestion with either *EcoRI* or *EcoRI* and *HindII* a 2% agarose gel was used to separate the fragments. Lanes 2, 4, 6, 9: *EcoRI* and *HindII* double digests of M13 mp2, M13 mp3, M13 mp4, and M13 mp1 respectively. Lanes 1, 3, 5, 8: *EcoRI* digests of M13 mp2, M13 mp3, M13 mp4, and M13 mp1 respectively. Lane 7: *HpaII* digest of M13 mp1. Fragment sizes are given in base pairs.

indicator plates³. From DNA sequence data⁴ and restriction endonuclease cleavage analysis (data not shown) it was known that an *EcoRI** site is located at the position corresponding to the fifth amino acid of β -galactosidase. This site should be converted into an *EcoRI* site by the change of guanine 13 into adenine as depicted in Fig. 1. In the amino acid sequence aspartic acid is then replaced by asparagine causing an increase of the net positive charge of the α -peptide with no major alteration in the shape of the primary side chain. The whole α peptide is basic, so the mutational change would be expected not to destroy the ability to undergo α -complementation, especially as an α -complementation-like restoration of mutant β -galactosidase can be mediated even by antibody against β -galactosidase (ref. 5 and I. Zabin, personal communication).

Alkylation of bases in a DNA molecule leads to mutations by several molecular mechanisms, one of which is mispairing⁶. Methylation of guanine at position O₆ has been shown to cause mispairing with uracil⁷. Therefore single-stranded (+) DNA of M13 mp1 phage was treated with *N*-[³H]methyl-*N*-nitrosourea, an alkylating agent, which has been reported to be effective for this type of methylation⁸, to produce two to four O₆-methylguanines per phage genome⁹. The alkylated DNA was used to transfect bacterial cells and allowed to undergo several cycles of replication. Supercoiled RF I DNA was isolated and digested by *EcoRI*. Linear molecules of genome length were separated from undigested molecules by agarose gel electrophoresis, excised from the gels and re-electrophoresed several times. Finally the molecules

were recircularised by way of their cohesive ends, treated with T₄ DNA ligase and recovered after amplification from transfected cells. DNA from single plaques was isolated and analysed as described in Fig. 2.

Following this procedure three individual clones with unique *EcoRI* restriction sites at different positions in the phage genome have been characterised. The frequency of *EcoRI* sensitive molecules in the phage pool after enrichment was about 1 in 10. From the known DNA sequence (ref. 4 and W. Gilbert, personal communication) and the restriction endonuclease cleavage analysis (Figs 2 and 3) two of them can be identified as the result of the conversion of the *EcoRI** sequences corresponding to the positions of amino acids 5 and 119 of β -galactosidase into the canonical *EcoRI* sequence respectively. The first mutant was denoted M13 mp2, the second M13 mp3, both of them mediate α -complementation. The latter mutant resulted from a change of base cytosine 355 of the β -galactosidase region into thymine by the presumed mispairing of imino-methylcytosine with adenine. The *EcoRI* site of the third mutant is located about 490 base pairs away from the unique *HindII* site in the *HpaII* C fragment. The mutation has therefore occurred in the genetic information for the 'X' protein and the distal part of gene II (Fig. 4). Since the DNA sequence of this region has not yet been determined the type of base change that led to this *EcoRI* site cannot be assigned.

The introduction of an *EcoRI* site into the *lac* structural part of M13 mp1 which itself is resistant to cleavage by *EcoRI* endonuclease creates a unique site to clone DNA fragments. It could be confirmed that insertion of *EcoRI* fragments into M13 mp2 leads to the inactivation or the reduction of the α -complementation ability since hybrid phages have been isolated as colourless or light blue plaque formers under the screening

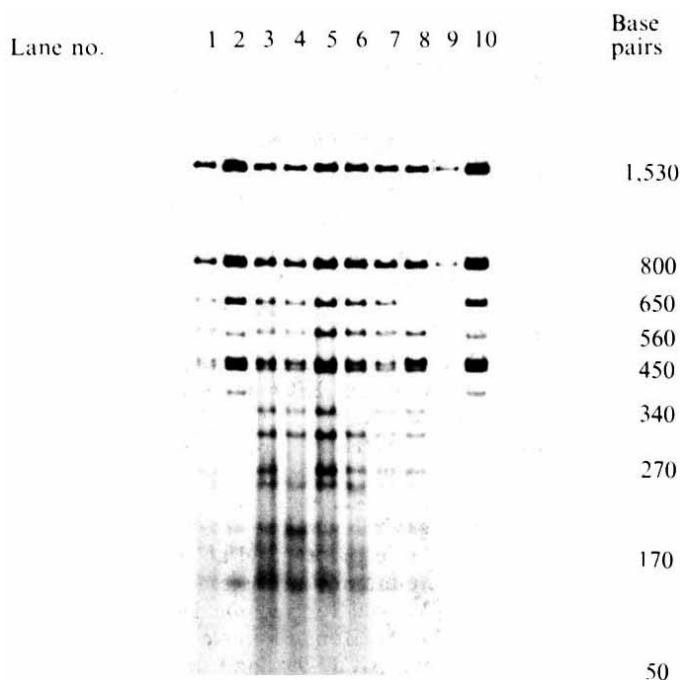


Fig. 3 Mapping of the three newly derived *EcoRI* sites by *HpaII*/*EcoRI* double digests. Purified RF I DNA of M13 wild type, M13 mp1, M13 mp2, M13 mp3, and M13 mp4 was labelled by nick translation, digested by *HpaII* restriction endonuclease (Biolabs) and *HpaII* plus *EcoRI* in the cases of M13 mp2, M13 mp3, and M13 mp4. Separation of the fragments was carried out on a 2% agarose gel. The sizes of the fragments are given in base pairs. Lanes 1, 9: *HpaII* digest of M13 mp1. Lanes 2, 10: *HpaII* digest M13 wild type. Lanes 3, 5, and 7: *HpaII* digests of M13 mp2, M13 mp3, and M13 mp4 respectively. Lanes 4, 6, and 8: *HpaII*/*EcoRI* double digests of M13 mp2, M13 mp3, and M13 mp4 respectively. In lane 4 the central *lac* fragment, *HpaII* G (see also Fig. 4), is missing which indicates that the *EcoRI* restriction site is located on this fragment; in lane 6 *HpaII* F fragment is missing assigning the *EcoRI* site of M13 mp3 to this fragment; lane 8 shows that *HpaII* C fragment is cut by *EcoRI*.

The mapping data from Figs 2 and 3 are summarised in Fig. 4.

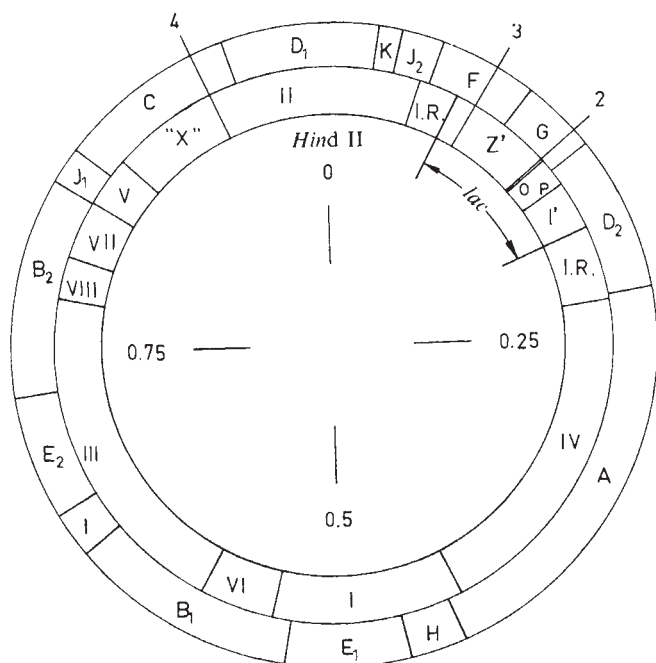


Fig. 4 Physical and genetic map of M13 mp1 and its derivatives M13 mp2, M13 mp3 and M13 mp4. Restriction fragments produced by *HpaII* endonuclease are indicated by capital letters. The genes are marked by roman numerals¹². The numbers 2, 3, and 4 denote the *EcoRI* restriction sites of M13 mp2, M13 mp3, and M13 mp4 respectively. IR intergenic region; X, X protein; I', part of the *lac* repressor gene; p, *lac* promoter; o, *lac* operator; Z', α region of the β -galactosidase gene.

conditions used. In addition, expression of functions coded by the inserted DNA can be controlled by the *lac* regulatory region. To improve the versatility of M13 mp1 phage vector other unique restriction sites and mutations can be derived by the method described here.

The alteration of DNA by base alkylation can be used not only for the creation of restriction endonuclease recognition sites but also for their elimination. In addition different types of base alkylation and mispairing may widen the application of this method to other specific base changes which are of interest in protein-DNA interaction.

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Note added in proof: From the DNA sequence determined by M. Schallers group we learned that the base change leading to M13 mp4 has occurred 490 bases away from the *HindIII* cut and is the same as illustrated in Fig. 1.

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Impaired gene activity for 18S and 28S rRNA in early embryonic development of mouse parthenogenones

ALTHOUGH many unfertilised mouse oocytes have been variously stimulated to develop into embryos—parthenogenones—all have died soon after implantation¹. The suggested causes of this failure include homozygosity of recessive lethals and an incomplete cortical reaction¹. It is not known whether the lack of the paternally-derived genome inhibits their proper development. Using selective silver staining^{2,3} we have examined the activity of the genes for 18S and 28S ribosomal RNA during preimplantation development of parthenogenones. From studies of the activity of nucleolus organisers on the chromosomes of human-mouse cell hybrids, it has been assumed that silver precipitation was a consequence of gene activity for 18S and 28S rRNA (refs 4–6). Our results indicate that these genes

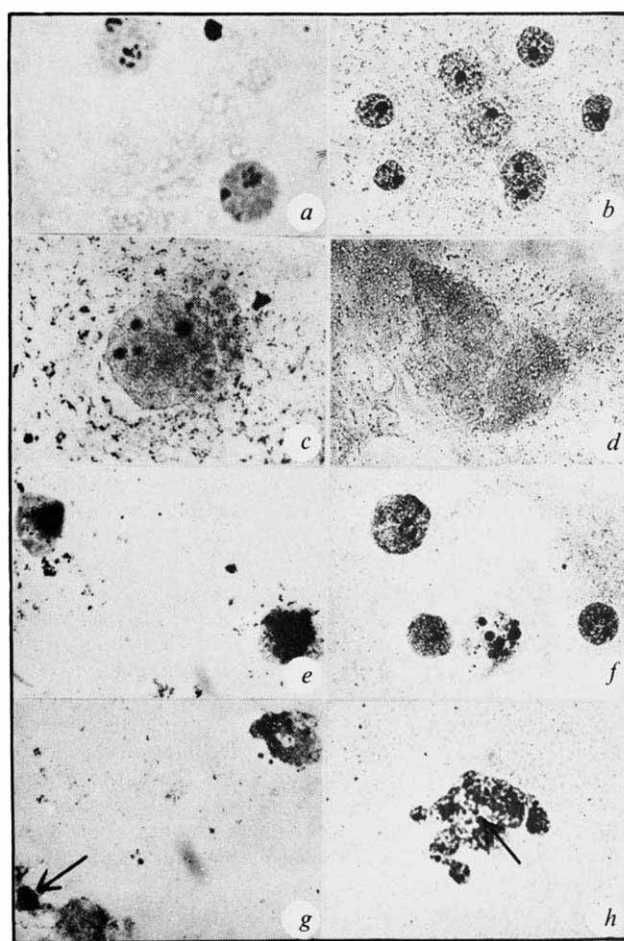


Fig. 1 Silver staining in mouse parthenogenones. A normal fertilised embryo at the 2-cell stage (a) and at the 8-cell stage (b) are shown for comparison. Note the prominent silver precipitation of the nucleus and of the cytoplasm of the 8-cell embryo. There is a high intensity of staining in the nucleus of a 2-cell parthenogenone (c) and the lack of any such nuclear staining in d, but with an unexpectedly high intensity of cytoplasmic staining in a parthenogenone with only one nucleus. Both parthenogenones (c, d) were prepared 43 h after electrical stimulation. A high level of silver precipitation is shown in two nuclei of a parthenogenone (e) with three nuclei 71 h after stimulation. There is a difference in the staining intensity of the various nuclei in the same 8-cell parthenogenone (f) 71 h after stimulation. No staining of the cytoplasm is visible. g, A 2-cell parthenogenone 42 h after stimulation. The persisting nucleolus (arrow) is heavily stained by silver nitrate. h, A morphologically abnormal nucleus 42 h after stimulation showing silver precipitation of some chromatin regions.

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Table 1 Onset of silver staining in mouse parthenogenones

Time (h) after electric shock	No. of eggs recovered	No. of eggs stimulated	Silver staining of nuclei	No. of stimulated eggs with											
				1	2	3	4	5	6	7	8	9	10	11	12 nuclei
15	22	13	+												
			—		8	5*									
31.5	8	7	+		2	2									
			—		2	1									
42	32	32	+		9	6	3	1							
			—		7	5	1								
61	7	7	+		1	2					3				1
			—												
72	27	12	+		4	2	2	2	2						
			—												

The parthenogenetic embryos are classified according to the time after electric stimulation, the actual number of nuclei observed, and the presence of nuclear silver staining.

* Eight eggs showed 1 pronucleus and five 2 pronuclei.

are activated during early embryogenesis and without the need for paternally-derived factors.

DBA/2 and BALB/c female mice were injected intraperitoneally with 6 IU pregnant mares' serum gonadotropin (PMSG) and, 48 h later, with 6 IU human chorionic gonadotropin (HCG) to induce ovulation. Stimulation of the tubal oocytes was achieved by an electric shock⁷ (40 V, 8 μ F capacitor) 20 h after the HCG injection. The mice were killed 15, 31.5, 42, 62 and 71 h after stimulation and their tubes flushed with Ringers' solution. The parthenogenones were transferred to 1.3% sodium citrate for 30 min, followed by fixation in methanol-acetic acid (3:1) on grease-free slides. They were then stained the same day with a 50% solution of AgNO₃ in distilled water (pH 5.0)⁸. The preparations were examined by phase contrast microscopy, and examples from different stages are shown in Fig. 1 a-h.

We observed the silver precipitation in nuclei of seemingly morphologically normal parthenogenones 31.5 h after electrical stimulation (Fig. 1, Table 1). No precipitation was visible in pronuclei 15 h after the electric shock. This observation is consistent with that of normally fertilised embryos⁸ when evidence of the first silver staining became apparent from the 2-cell stage onwards, but not in the ovulated oocytes or pronucleus stages on the first day after fertilisation. The amount of silver precipitation increased from the 2-cell stage to 8-16-cell stage in fertilised embryos (Fig. 1). This correlation was not found in parthenogenones, but a good correlation was found with the developmental age, irrespective of whether cleavage had been delayed or not.

This apparent dependence on the developmental age, rather than on the actual stage of development, would probably also explain the initial variation at the onset of nucleolus reactivation in other species. Mouse embryos reactivate their nucleoli between the 2-cell and 8-cell stage, whereas rabbits do so in late morulae, that is, 68-128 cells⁹. Reactivation, however, occurs in both species at almost the same time, namely, 48-60 h after fertilisation. This, together with our observation of mouse parthenogenones, rather suggests that the onset of gene activity for rRNA might depend entirely on the time interval following electric stimulation or fertilisation.

Some parthenogenones were delayed in their development. These, and those with morphologically abnormal nuclei, showed an impaired silver precipitation (Fig. 1 f-h). The better developed parthenogenones, however, showed an intensity of silver precipitation comparable with that of normal embryos. Nevertheless, we did observe, even in non-delayed stages,

apparent differences between single blastomeres of one and the same parthenogenone (Fig. 1 f). Such impairment of silver precipitation within one parthenogenone may indicate a disturbed regulation of NOR activity, or a gene-dosage effect for 18S and 28S rRNA caused by a variable number of NOR chromosomes within the nucleus. The latter suggestion would not altogether be unlikely, because chromosomal aneuploidy is a common feature in these embryos¹⁰.

Our observations with silver nitrate indicate that the onset of gene activity for 18S and 28S rRNA in apparently morphologically normal mouse parthenogenones is similar to that in fertilised embryos. Gene activation of this type of RNA depends, therefore, not on factors transmitted by the sperm alone, but on storage products (preformed molecules within the cytoplasm of the oocyte) or on products synthesised at a given time after ovulation, stimulation or fertilisation. This type of regulation would therefore depend solely on maternally derived factors. Impaired activity for 18S and 28S rRNA might be one of several causes for the early loss of mouse parthenogenones.

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Non-rejoining DNA breaks and cell inactivation

RITTER *et al.*¹ measured non-rejoining DNA strand breaks (NRBs) produced in V79 hamster cells by radiations of different linear energy transfer (LET) and found a correlation between the efficiencies of NRB production and low-dose 'single-hit' cell inactivation. However, we suggest that this comparison is inappropriate. Radiation survival curves are commonly interpreted² as a mixture of irreversible lethal interactions following single-hit kinetics (leading to the initial slope on curves of log survival against dose) and sub-lethal interactions following multi-hit kinetics to give a slope which increases with dose before reaching a constant final slope. For low LET radiations the single-hit interactions dominate at low doses ($\lesssim 400$ rad in these cells) and the multi-hit interactions at high doses. Since Ritter *et al.*¹ measured NRBs at doses of 3,000 to 4,500 rad there seems to be no justification for their comparison with initial (low dose) slopes of survival curves.

An appropriate comparison must be between the efficiency of NRB production and survival curves at as high a dose as possible. This is given in Fig. 1a for the established human T1 cells^{2,3} cited by Ritter *et al.*¹. The agreement in relative efficiencies is poor, but this is expected when the average number of lethal events produced by a single ion passing through a cell is large. Cell inactivation measurements will underestimate the number of lethal events because one or more lethal events per cell will be detected as only one inactivation. When allowance is made for this (see Fig. 1 legend) there is good agreement between the production of NRBs and lethal events at high doses (Fig. 1a), except at the highest LETs where the correction is very sensitive to small alterations in projected nuclear area and the presence of δ -rays. On this basis the low-dose relative efficiencies for the production of lethal events are much higher than those cited by Ritter *et al.*¹ and the apparent agreement they found may be fortuitous since they made no allowance for the underestimation of lethal events.

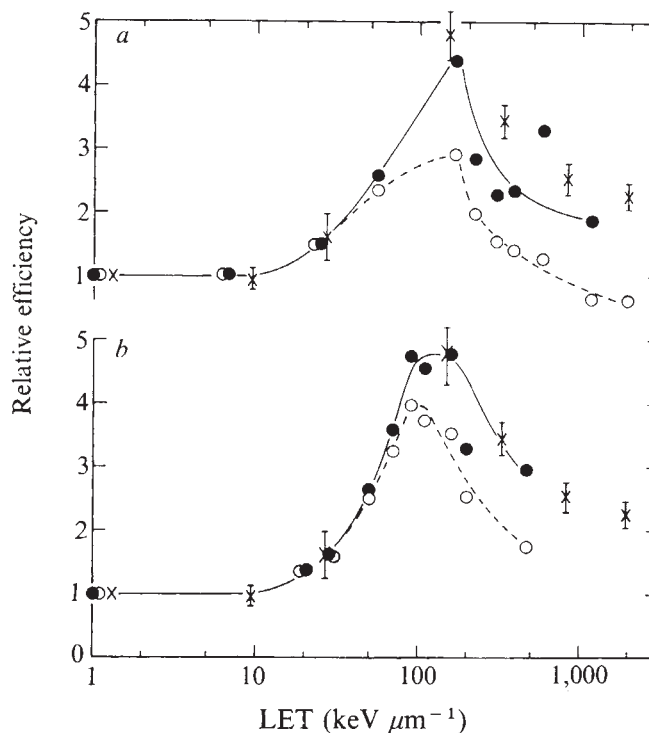


Fig. 1 Relative efficiencies of non-rejoinable DNA break production¹ (×) and of mammalian cell inactivation (○, ●) by radiations of different LET. *a*, ○, Inactivation of T1 human kidney cells derived from the final slopes (λ) of $\ln$ survival-dose curves using Todd's fitted parameters². The radiosensitivity λ can be related to the average number of lethal events (x) produced by a single ion passing through a cell by^{5,6}

$$\lambda = (A/16L)(1 - e^{-x})$$

where L is the LET in $\text{keV } \mu\text{m}^{-1}$ and A is the projected area of the sensitive portion of the cell in μm^2 . This equation follows from the assumptions that the distribution of lethal events produced by a single track in passing through A , and the distribution of numbers of tracks through A , are both poissonian. Then the probability of a cell having no lethal events (that is, surviving) after the passage of an average of $m = AD/16L$ tracks is

$$S = \exp[-m(1 - e^{-x})]$$

and the radiosensitivity is

$$\lambda = \frac{d(\ln S)}{dD}$$

Hence the relative efficiency for production of lethal events (per unit dose) can be deduced for each radiation. Data are shown (●) for $A = 100 \mu\text{m}^2$ which corresponds approximately to the mean projected nuclear area of these T1 cells^{2,3}. *b*, Inactivation of freshly isolated human fibroblasts⁴ (○) derived from the slopes (λ) of their purely exponential survival curves; (●), production of lethal events for $A = 150 \mu\text{m}^2$ (the approximate mean projected nuclear area of these cells). In all cases the X-ray points have been plotted at a nominal LET of $\sim 1 \text{ keV } \mu\text{m}^{-1}$ for convenience.

Our own data on the survival of V79 cells⁴ give similar agreement between the production of NRBs and lethal events at high doses. The final slope of two-component low LET survival curves for established cell lines

often cannot be determined accurately, but curves for freshly-isolated human fibroblasts show little or no change in slope and the unique slope may be estimated over a large dose range. Figure 1b shows our data⁴ for these

cells and the efficiency of NRB production is in excellent agreement. This analysis endorses the view that there is a relationship between the production of lethal events and NRBs measured on alkaline sucrose gradients, but there is not a 1:1 correlation. A calculation based on the above data and assuming that each cell contains about 6 pg of DNA shows that ~20 NRBs correspond to 1 lethal event.

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RITTER ET AL. REPLY—Goodhead *et al.* state that the initial slope mechanism of cell killing is dominant only at low doses, so that high-dose, non-rejoining DNA strand break (NRB) data cannot be compared with it. Such a comparison can be made, however. According to the model, the initial slope, single-hit mechanism is equally operative at all dose levels. It is not particularly a low-dose phenomenon, but is measured at low doses simply because there its effect is not masked by multi-hit modes of cell killing.

Furthermore, we have found, using high doses, that NRBs are induced linearly with dose (the percentage of NRBs is constant with respect to dose¹). If it is assumed that this linear relationship can be extrapolated to the low doses associated with high survival, then the NRB induction efficiencies that we measured are valid at low dose levels.

Thus, either of the two above arguments indicates that it is valid to test the hypothesis that single-hit cell killing is related to NRB induction by comparing low dose slopes of survival curves with NRB data obtained using high doses. Multi-hit, sublethal damage is presumed, here, to be the result of some other lesion(s).

Goodhead *et al.* stress the need for a correction for underestimation of lethal events in cells exposed to high-LET particles. We agree. They compare corrected "final slope" survival data with our NRB data and find a reasonable agreement between the two.

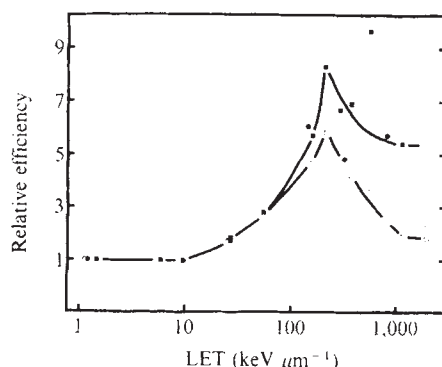


Fig. 1 Relative efficiency-LET relationships for single-hit (initial slope) cell killing and NRB induction. Open symbols, uncorrected data; closed symbols, data corrected as described below. Single-hit cell killing efficiencies of Todd for T-1 human kidney cells^{4,5} are plotted uncorrected (□) or corrected (■) for underestimation of lethal events. The correction is carried out as for Fig. 1a of Goodhead *et al.*⁹. NRB induction efficiencies¹ are plotted uncorrected (○) or corrected (●) for underestimation of such breaks in alkaline sucrose gradients. In the conditions used single-strand pieces of DNA of less than about 1×10^6 molecular weight cannot be detected. Because of this resolution limit and the limited radial extent of ionisation about the particle track, probably only one break can be detected per track—DNA intersection. To correct for this, a procedure similar to the above was used. A good fit of corrected NRB data to single-hit-killing data was obtained assuming an NRB target diameter of 27 Å, agreeing closely with the diameter of double-stranded DNA.

Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

We feel, however, that the use of the final slope as a measure of cellular inactivation at various LET's is problematic because: (1) the status of the final slope as a fundamental measure of cell inactivation is somewhat uncertain. A true final slope does not apparently exist for many established

cell lines^{2,3}, including the T-1 cells used by Todd^{4,5}, but may exist for human diploid lines⁶ (also our own unpublished data). (2) It has been clearly demonstrated that initial slope, single-hit-like processes are predominantly responsible for the variation of cell killing efficiency with LET^{4,7,8}.

Therefore, we have again compared Todd's initial slope data^{4,5} with our NRB data, this time correcting both sets of data for underestimation of events (Fig. 1). The correction for Todd's data is carried out as described by Goodhead *et al.*⁹, while our correction for NRBs arises for a different reason—in those conditions in which alkaline sucrose gradients are used to measure the residual DNA strand breaks (NRBs) after long repair times, single-strand pieces of DNA of molecular weight less than 1×10^6 are not resolvable. High-LET, heavy ions are likely to induce breaks at intervals smaller than this (see Fig. 1 legend). It is seen (Fig. 1) that after these corrections, there is, as before, good agreement between Todd's initial slope data and our NRB data. We therefore maintain our view¹ that non-rejoining DNA strand breaks may be causally related to single-hit, initial-slope cell killing. Assuming 6 pg of DNA per cell, a calculation based on the above data indicates that about 50 NRBs per cell correspond to 1 lethal event by the single-hit mechanism.

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reviews

Aquaculture treatise

J. H. S. Blaxter

Marine Ecology: A Comprehensive, Integrated Treatise on Life in the Oceans and Coastal Waters. Edited by O. Kinne (Wiley: Chichester and New York.) Vol. 3: Cultivation. Part 1: pp. xiii + 577. (1976.) £22.05; \$41.05. Part 2: pp. xv + 578–1293. (1977.) £29.50; \$59. Part 3: pp. ix + 1294–1521. (1977.) £15; \$30.

THE cultivation of aquatic organisms has been practised for centuries but the increase in the human world population from about 10^9 in the eighteenth century to about 4×10^9 at the present time with a projected $9\text{--}16 \times 10^9$ by the mid-twenty-first century has given impetus to the artificial improvement of food resources. It is sad to report (p1341) that aquaculture is unlikely to contribute more than 1.4–2.1% of the global annual protein yield by the end of the present century. Even this yield requires the utilisation of much more coastal land and improvements in technology.

Professor Otto Kinne from the Biologische Anstalt Helgoland continues his marathon editing work with a contribution to this technology. The three parts (each a separate book) to Volume 3 are on aspects of cultivation. Volume 1 (Environmental Factors) with three parts and Volume 2 (Physiological Mechanisms) with two parts have already seen the light of day over the past nine years. With two volumes to go (4 on Dynamics and 5 on Ocean Management) and a separate book on Diseases of Marine Animals, we shall eventually have the marine ecology equivalent of the *Handbook of Sensory Physiology*.

The reader will at once notice a remarkable fact—of the total of 1521 pages in the three parts Professor Kinne has himself contributed about 1000 pages. His versatility and energy also show in earlier volumes, in which he has contributed chapters on such a wide range of topics as temperature effects and the ecology and physiology of marine mammals.

The series maintains a high standard of presentation—no downgrading yet by the use of photo-offset processes. The three parts of Volume 3 are full of factual information in the form of tables, figures, good bibliographies, and subject, author and taxonomic indexes. I find this general informative style exceptionally useful in making the books a source of reference rather than sustained reading.

Part 1 contains a long chapter by

Kinne on water quality management and technology which includes sections on culture systems and enclosures, treatment of water, especially filtration, and detailed descriptions of aquarium and culture equipment of all kinds. This chapter comprises a unique collection of information of use to hundreds if not thousands of marine biologists. The remainder of Part 1 consists of chapters on culture of bacteria by K. Gundersen (Sweden), fungi by J. Schneider (Germany), yeasts by H. G. Hoppe (Germany), unicellular plants by R. Ukeles (USA) and multicellular plants by S. Bonotto (Belgium).

Part 2, written by Professor Kinne, treats the research cultivation of representatives of each phylum of animals from Protozoa to Mammalia, considering not only the conditions for successful rearing but also features such as gamete storage, transport of stock, induction of spawning and nutritional requirements. There are over 100 pages of references alone. Part 3 picks out some special features: axenic cultivation by L. Provasoli (USA), commercial cultivation (aquaculture) by O. Kinne and H. Rosenthal, multispecies cultures and micro-

cosms by M. Levandowsky (USA) and chemical contamination of culture media by M. Bernhard (Italy).

The three parts must be seen as a continuing volume. It is not possible to recommend them separately so that libraries or a few individuals must pay a total of £63. In my view they are, by present day standards, getting value for money.

It may seem ungracious to find fault with such a major work. The presentation probably is a little stereotyped with little author individuality showing through. The editor may have attempted to spread his net too wide. There is no doubt some of the subject matter is not really marine ecology at all, but a treatment of all aspects of life in the sea and in captivity.

The editor's task in holding authors to deadlines must be a difficult one. And by the time Volume 5 is published in the late 1970s or early 1980s Volume 1 will be grossly out-of-date. Will the editor ask the earlier authors to bring their chapters up-to-date? □

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Human nutrition and crop production

Plants, Food and People. By M. J. Chrispeels and D. Sadava. Pp. 278. (Freeman: San Francisco and Reading 1977.) Hardback £9.60; \$12; paper £4.50; \$6.

THIS book, which is aimed specifically at the "informed lay-person", sets out to provide some account of the scientific bases of human nutrition and of crop production, laying emphasis on aspects related to the problem of feeding the growing world population.

Following an introduction dealing with population and some aspects of food use it goes on to discuss some principles of human nutrition. This is followed by chapters on energy and crop production, plant nutrition, and some aspects of agricultural practices; and the book concludes with chapters on pest and disease control, plant breeding and the Green Revolution, alternative food sources, and future prospects. The canvas is therefore vast and I wonder if it is really possible to tackle it with uniform success in all areas with an audience whose previous expertise is as ill-defined as that of the "informed lay-person".

The more general parts of the text are well written and informative and the selection of examples is good. The tables and diagrams are clear and helpful, though an indication of the scatter of points about some of the idealised curves presented would have improved confidence in these. In the later parts of the book, some of the material is very stimulating and the discussion on the Green Revolution and its implications is clear and avoids euphoria. I felt it a pity that the ethical issues clearly implied were not tackled at greater length in the final chapter, which was disappointingly short.

The problem of the identity of the audience is greatest in the more technical parts of the book where, although every effort is being made to avoid jargon, some of the terminology would be bewildering to a non-scientist. At the same time it is almost impossible in such texts to avoid irritating over-simplification. For example, "if the top bud is removed the top (of the stem) will branch" (p53). "Potato tubers develop buds which sprout; the sprouts are planted" (Fig. 3.10; incident-

ally the structure illustrated surely cannot be a potato tuber?).

The book is well produced and mostly well illustrated. I found it interesting and, as is often the case with cross-disciplinary texts, helpful in areas where my knowledge was slight. For a person of the right background it could well be very useful general reading, though the serious student would probably be irritated by

the very sparse referencing. This is a characteristic it shares with a number of modern texts, and in a book well laden with facts the inability of the reader to gain direct access to sources is regrettable. The hardback seems over-priced at £9.60.

T. A. Hill

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Geological atlas

Geological World Atlas. 1:10,000,000. (Commission for the Geological Map of the World.) (UNESCO Press: Paris, 1977.) Ffr. 650.

THE first six sheets of the *Geological World Atlas* have been published. Two cover North and Central America, three cover Africa, and the sixth sheet covers the Pacific Ocean. There will be 22 sheets in all; the remainder are expected to be ready before the end of 1980.

The continental maps are on a geographical base of 1:10,000,000 provided by the American Geographical Society; the Pacific Ocean is at a scale of 1:36,000,000. Much of the ocean floor bathymetry was provided by the Soviet Academy of Sciences. The projection used for America is the bipolar oblique conical conformal projection; that for the remaining sheets is the conformal Miller oblate stereographic projection. The maps were compiled at UNESCO in Paris and printed in Australia.

A short explanatory text in French and English outlines the most significant geological features of a region, describes the principal sources of the data and gives a useful reference list. The sheets show and name the principal rivers and capital cities, but all other cities are shown by initial only. Political boundaries and the names of countries have been omitted. Oceanfloor topography is shown at 200 m, and 1, 2, 3, 4 and 5 km.

Technically the maps are excellent: the colours are bright, clear and in register. Scientifically, they are a considerable achievement. The stratigraphic subdivisions are well placed; the scales and projections judiciously chosen. The maps reveal features that might otherwise escape the eye. For example, most geologists have some idea of the outcrop area of the Columbia River lavas in the western United States. But fewer will know that the outcrop areas of lavas in western Mexico, Honduras and Nicaragua is much greater—a fact immediately apparent from the Atlas. By turning a page, one may also see that the Lower Jurassic lavas of southern Africa were once of comparable extent.

In the Pacific map, the age pattern of

the oceanfloor is clearly revealed at a glance. The oldest, of Upper Jurassic age, lies next to the Marianas. West of the Marianas lies one of the several subduction-coupled basins of the western Pacific, all of which are Cenozoic in age (unless one includes the oceanfloor between Lord Howe Rise and Tasmania). The systematic changes in the ages of the Hawaiian–Emperor volcanic chain is clear, as are similar but less well-known changes in the innumerable islands to the west.

Reproductive biology

Reproduction. By J. Cohen. Pp. 356. (Butterworths: London, 1977.) Paperback £4.95.

To attempt a description of virtually all aspects of reproduction throughout the whole Animal Kingdom in the space of 330-odd pages is a formidable task, especially when attention is given to such peripheral topics as the arithmetic of reproduction, the evolutionary significance of larval forms, and the reproduction of human cultural patterns, in addition to the expected range of material. This is evidence of the author's strongly individualistic approach to the subject of reproduction, for which there are many other indications throughout the work, and which contributes in a major way to its readability. The aim has been to show how reproductive biology, despite its very interdisciplinary nature, can be shown to constitute a single integrated body; and to a noteworthy degree the goal is attained.

Much is achieved by a broadly comparative approach to a subject like reproduction, which must necessarily have been a major function of living organisms from the beginning. This approach is used consistently and the book starts with a consideration of general issues, such as kinds of reproduction, the role of cell division, the theory of the germ plasm, and the influence of selective processes in reproduction, and passes on to methods of aggregation of sexes and germ cells (with some thought on restraints in animal and human societies).

The morphology of reproductive organs, the germ cells and the fertilisation

Minor criticisms were noted: the fold-out maps do tear easily where tucked in. This is unfortunate, and should not happen in such a volume. The volume is expensive and is unlikely to reach as large an audience as it deserves to, particularly when library funds are so limited. The transform faults in the Pacific map are somewhat diagrammatic.

The publication of the completed Atlas will be a splendid achievement. The foreword outlines some of the previous attempts, begun well over a century ago, that culminate in the international compilation of the present *Atlas*. It is certain that geologists with global interests will spend many hours poring over the maps. Perhaps they can look forward to a Tectonic World Atlas to complement this one?

A. G. Smith

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process receive detailed treatment, but the statement that uncapacitated spermatozoa of man, guinea pig and wallaby can penetrate and activate eggs is misleading and reveals some unfamiliarity with the data. The role of the maternal environment on early development is set out interestingly, though it is questionable whether some of the influences thus ascribed are strictly maternal in origin, and the author errs in implying increase in cytoplasmic mass in the mammalian cleavage embryo. The mechanisms of gastrulation and neurulation are described carefully and some time is spent on the ways in which the zygote genome finds expression.

Attention is then switched to wider issues—the developmental and evolutionary significance of larval forms (in which children make a curious appearance), the relative advantages of viviparity (including ovulation rhythms, nidation, placentation and birth), and the care of offspring (which brings together such diverse topics as egg yolk, milk, protection and education). The last four chapters are concerned with life cycles, regulation of numbers, reproduction and evolution, and reproduction in mammals. The final chapter is essentially concerned with hormones in reproduction, but includes also even some sociology.

This is a remarkable compilation of data, highly ambitious but on the whole dextrously and vigorously handled, so that the reader receives not merely a useful introduction but also a stimulus to thought. Its main appeal will be to the comparative biologist, whether student or teacher, and for them it can be warmly recommended.

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Zellkern und Zellzyklen

Zellkern und Zellzyklen. By Walter Nagl. Pp. 486. (Eugen Ulmer: Stuttgart, 1977.) DM120.

THE literal translation of the German title of this book would be "Cell nucleus and cell cycles". However, in my opinion this is far too modest a title considering the book's scope. A short glimpse at the table of contents convinces the reader that the book has a much broader outlook and deals with such fundamental problems as molecular, structural and functional organisation of the cell nucleus and chromatin; DNA replication, mitotic and various modified cell cycles; and the role of the cell nucleus, nucleo-cytoplasmic interactions and cell cycles in development and differentiation of multicellular organisms. All these topics are covered from a development biologist's point of view. The author is a well known cell biologist who originally carried out research in plant cytology and discovered the polytenic chromosomes in the suspension cells of the plant *Phaseolus coccineus* in the early sixties. At this time it was still fashionable for cytologists as well as for cell biologists, trained either in botanical or zoological sciences, to have only a very limited mutual exchange of ideas. Nagl was certainly one of the few 'botanists' who realised very early that such historical and in many respects artificial boundaries between such disciplines had to be transgressed in order to solve the fundamental and really important problems in biology.

The book is part of a series entitled "*Phytology—Classical and Modern Botany Represented by Individual Scientists*". One can pose the question as to whether the author possibly succumbed somewhat to the concept of this series. I found that the botanical cell and molecular biology is treated very comprehensively but that important results and concepts derived from molecular biology of zoological or human cell material are either not discussed or only incompletely. Although Nagl proves to have an extremely broad knowledge of botanical and zoological developmental biology, I feel that it is impossible for any single scientist to cover the whole field of molecular and cellular developmental biology of plants, animals and humans really authoritatively.

The author, however, has done an immense job, considering that nearly 4,000 papers are cited in the book. This naturally makes it an excellent reference book, especially for someone who lacks an extensive knowledge of either plant or animal cell and molecular biology. On the other hand, so many citations create difficulties not only for the writer but also

for the reader. Personally, I often missed the emphasis on important and fundamental facts and a critical evaluation of different and/or conflicting experimental results and interpretations, and for this reason I would not recommend the book as a textbook for undergraduate students.

The book contains not only experimental results but also gives a full description of many experimental techniques used in cell and molecular biology, for example, DNA, RNA and chromatin isolation. Since only standard techniques are presented, the reader should bear in mind that generally such techniques have to be modified and adapted for different animal and plant species. The many informative tables which summarise data on cell and molecular biology from a large number of plant and animal species deserve special attention.

Individual differences in human memory

Human Memory: Theory, Research and Individual Difference. By Michael W. Eysenck. Pp. 366. (Pergamon: Oxford and New York, 1977.) £8.

HUMAN MEMORY has been a popular topic for books in recent years; some are general texts, at elementary or advanced levels; some concentrate on special areas; and some are monographs on recent research. This particular book has elements of both the latter classes. The core of the work is formed by the author's own studies of individual differences in memory; these are fleshed out by chapters reviewing each of the areas of memory closest to those studies. There is a notable concentration on the most recent and little-known findings; references to 1977 are common; and quite a few of the experiments cited are still unpublished. As a result, these general review chapters are likely to be useful and suggestive reading for research workers active in topics such as the use of imagery, retrieval mechanisms, or sentence memory. They will find much that is new to them. Conversely, some of the experiments quoted from other workers may be hard to replicate, and many promising ideas are still untested. Because of this, because the relative absence of old-established findings or those from other approaches to memory, and because there are rather few links between the particular fields of research, one would scarcely advise this book as a general textbook. Specialists, however, ought to read it and will probably find particular sections that they will want their students to read.

The main glory of the book is the emphasis on differences between people. That topic is long neglected by experi-

We all know that progress in cell and molecular developmental biology has been extremely rapid in recent years, accompanied by an ever-increasing number of publications. The author has only cited literature up to autumn 1975, and it is therefore understandable that important new results and interpretations, for example, on chromatin structure, DNA sequencing or recombinant DNA research, are not discussed. In summary, I consider Nagl's book as a very valuable source of reference for any scientist who is interested in the structure and function of the cell nucleus, chromatin and DNA from a developmental biologist's point of view.

Heinz Tobler

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mentalists despite the reminders of its importance which are to be found in the raw data of every study. The particular line taken by this author is to review the evidence for changes in memory produced by temporary changes in the state of arousal, to regard the degree of introversion or neuroticism of the individual as resulting in chronic differences in arousal, and then to examine differences in memory between people differing on the personality dimensions. The hope is to find evidence of changes like those produced by arousal within an individual, and a number of encouraging results are indeed reported. There are also brief accounts of the effects of ageing, of "field dependence", of intelligence, and other similar factors. Given the general neglect of individual differences, however, the main focus is on the implications for memory of the two major dimensions of personality.

As those in the area will know, there are severe problems in linking arousal too simply to those dimensions. The formulations of Gray, of Pribram, or of other authors are quite different from those of the older Eysenck, and are not given detailed analysis here.

One can well accept the experiments of the son, and indeed the two-dimensional data of the father, without accepting the theoretical structure of the latter. Many of us therefore will welcome the revival of individual differences in memory, will feel that every research worker in the field should read the findings in this book or in the author's earlier papers; and yet feel the need to link those results to a different class of theory. Meanwhile, the results themselves are a worthy step forward.

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Stability of the fluvial system

The Fluvial System. By S. A. Schumm. Pp. 338. (Wiley: London, New York and Toronto, 1977.) £16.50.

THE knowledge of fluvial matters advances so rapidly that some aspects of it require re-appraisal every decade. Yet there still remain a few fundamental aspects which need stating fully anew, among them the inherent stability or instability of the fluvial system as a whole. This integral approach has increased in importance in recent historic times in proportion as the activities of mankind have impinged upon natural processes and caused unexpected reactions. Dr Schumm is a recognised authority on fluvial backwash and here takes the opportunity of incorporating some of his hitherto unpublished observational and experimental studies on the problem.

Wisely refraining from trying to outdo existing large textbooks on open-channel hydraulics, geomorphology, sedimentology and stratigraphy, he presents an account of concepts on the internal stability or instability of drainage basins. He provides details of the validity of these concepts and expounds on their wide practical applications. As would be expected, his approach is refreshingly empirical and stems from a true realisation of the sort of practical problems that all too often face civil engineers, economic geologists and any other Earth scientists who tamper with the Earth's surface.

The practicability of the text is increased by emphasis throughout on features and events of moderate scale and duration or on intermediate spans of time that tend to be neglected by geologists and geomorphologists who so often look more for long-term effects. Thus, landforms are essentially ignored to make space for details on, for example, changes in channel character and the distribution of sediment types in valleys, fans and alluvial plains.

The fluvial system is viewed—as it should always be—as a unitary, indivisible whole, and the text is organised to leave a complete fluvial picture in the reader's mind. After brief introductory

chapters on the general geomorphic concepts and the variables and rates of change in fluvial systems, there follows an excellent chapter on climatic change and paleohydrology, both Quaternary and earlier. The main body of the text (pp57–320) contains a tripartite analysis of the drainage basin, starting with the drainage divide or sediment source area, and proceeding through valleys and valley fills in the transfer zone, to depositional sites on the piedmont and coastal plain. The concepts and detailed arguments are summarised in a concluding chapter on the fluvial system, particularly from the point of view of soil conservationists, land managers, river engineers, geomorphologists and geologists.

Seemingly anomalous features of the fluvial landscape or their erratic development through time are explained by three concepts: intrinsic geomorphic thresholds; complex response; and episodic erosion and deposition. These, the author

contends, provide a means of developing a better understanding of the dynamics of landform evolution and, perhaps more importantly, a means of identifying within a landscape those components of the system that are inherently or incipiently unstable but are amenable to man's control. The same approach would also form a basis for predicting the future behaviour of existing unstable landforms, which is one of the major aims of modern geomorphologists.

This volume, with its generous illustrations and lists of latest references, will be welcomed also by Earth scientists generally. It fills an important niche in fluvial literature, and its lucid style and clear exposition place it well within the range of students in any form of higher education.

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Research on the eukaryotic chromosome

The Eukaryotic Chromosome. By C. J. Bostock and A. T. Sumner. Pp. 525. (Elsevier/North-Holland: Amsterdam, New York and Oxford, 1977.) \$79.75; Dfl. 195.

THE cell nucleus, once seen as a bag of loosely-organised material, is now emerging as a highly structured region: "A place for everything, and everything in its place", to quote Samuel Smiles out of context. This book brings together the results (up to the end of 1976) of the most recent wave of chromosome research, which has originated partly from the sophistication of techniques for the analysis of their molecular biology, and partly from the discovery of their gross substructure, as manifest by the various banding techniques. There has not hitherto been (as the authors discovered to their surprise) a comprehensive and up-to-date review of the subject. There is admittedly no right moment to bring out any review of a rapidly expanding field; and important discoveries will inevitably be made before it appears in print—in this case most notably the recent discovery that genes are not continuous but contain inserts of material of unknown function. But the book is as complete and up-to-date as is possible with a work of such high quality.

The authors collate information from any research on eukaryotic chromosomes which sheds light on their fundamental anatomy and physiology. It does not try to deal with comparative chromosomal

studies in relation to speciation, chromosomal pathology in man, or the details of gene assignment to human chromosomes (although the principles underlying this are discussed); this information is available elsewhere. Nor do they deal with every variant of the eukaryotic chromosome (for example, the microchromosomes of birds are not mentioned), although the specialised polytene and lampbrush chromosomes are considered in great detail because of the light they shed on chromosomal function.

The book starts with an introduction which includes the fundamental C-value paradox (why so little of the DNA present can be genetically active). Chapters follow on chromosomal DNA; RNA and proteins, and their molecular interaction in chromatin; the interphase nucleus in relation to spatial organisation; relationship of chromosomes to nuclear membrane; chromosome replication; polytene chromosomes; mitotic and meiotic chromosomes; and lampbrush chromosomes. The last four chapters cover substructure and banding, location of DNA sequences and genes, damage and repair, and models of chromosome structure.

The book is clearly and homogeneously written; each chapter starts with an introduction for the uninitiated. It is comprehensive, accurate, and produced to the highest standard. There are many diagrams and photographs (including electron micrographs), and these are beautifully clear. Inevitably, the price is enormous. But it pays to buy the best, and this is it.

E. H. R. Ford

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● In the review of *The Lymphocyte* (*Nature*, 9 March, 272, 193; 1978), line 4 should read: "it is easy to judge harshly as it", and not as shown.

Recent scientific and technical books

Applied biology

- BUNN, H. Franklin, FORGET, Bernard G., and RANNEY, Helen M. *Human Hemoglobins*. Pp.xii + 432. ISBN-0-7216-2178-3. (Philadelphia, London and Toronto: W. B. Saunders Company, 1977.) £17.
- CAMPBELL, Robert A., MORRIS, David R., BARTOS, Dagmar, DAVES, G. Doyle, and BARTOS, Frank (edited by). *Advances in Polyamine Research*. Vol. 1. Pp.xviii + 282. ISBN-0-89004-189-X. \$33. Vol. 2. Pp.xvii + 377. ISBN-0-89004-194-6. \$39. (New York: Raven Press, 1978.)
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obituary

Bernard Gregory, 1919–1977

BERNARD GREGORY died on 24 December 1977, the victim of a heart attack at the age of 58. His untimely passing is a great loss for the European scientific community. Active in both teaching and research, he mainly became a great scientific administrator, excelling in a role which assumes particular difficulty and importance now that scientific research demands large resources, often concentrated in big laboratories or managed on a national scale.

He was marked out by an intellect of the highest order and a deep sense of justice. His sure judgement, the result of exceptional gifts of analysis and synthesis, coupled with the simplicity and warmth with which he knew how to listen and to persuade, was the key to remarkable accomplishments at European level in the organisation of research in high energy physics, the field of his own original work. The European Organization for Nuclear Research (CERN) benefited particularly from his influence: he was its Director-General from 1966 to 1970, and, having been elected President of the Council of the Organisation, was to have taken up his duties in January 1978. In France, as Director-General of the Centre National de la Recherche Scientifique from 1973 to 1976 and later as Délégué Général à la Recherche Scientifique et Technique, he had a profound impact on many aspects of the scientific life of his country.

Bernard Paul Gregory was born at Bergerac on 19 January 1919. In 1938 he entered the Ecole Polytechnique in Paris, but his studies were interrupted by the war. He was taken prisoner in 1940 and remained in captivity for the duration of the conflict. After returning to Paris, he graduated top at the Ecole Polytechnique and qualified as an engineer in the Corps des Mines. He was drawn to scientific research and spent three years at the Massachusetts Institute of Technology, working under Professor Bruno Rossi on nuclear reactions caused by cosmic rays. He defended his doctoral thesis in 1950.

Back in Paris, he devoted himself to fundamental research, working at the Ecole Polytechnique laboratory directed by Professor L. Leprince-Ringuet. In this classical period of the study of cosmic rays with Wilson cloud chambers, the team led by Gregory and



Charles Peyrou built one of the most powerful detectors then known, with two cloud chambers in tandem, and set it up for operation on the Pic du Midi in the Pyrenees. With it, a large number of results were obtained on the decay of strange particles, notably the demonstration of the muon-neutrino decay mode of the K meson, whose existence played an important part in the understanding of the weak interactions.

This type of research, however, was shortly to undergo a radical change with the advent of the big particle accelerators. Bernard Gregory was the architect of this change in the laboratory of the Ecole Polytechnique. After a year spent at the Brookhaven National Laboratory, in the United States, to master the technique of bubble chambers, he took in 1958 the initiative for the construction of a hydrogen chamber 81 cm in length. This detector, one of the most powerful of its day, was built at Saclay and installed at CERN, in Geneva, where the 28 GeV proton-synchrotron, the PS, had been commissioned in 1959. Between 1961 and 1971, the 81 cm bubble chamber was to provide over 16 million photographs for the benefit of a number of European laboratories. Bernard Gregory played an active part in the scientific exploitation of the detector, which was succeeded at CERN by larger detectors, most recently the two

giant chambers now mostly devoted to neutrino physics — Gargamelle, also built at Saclay and the Big European Bubble Chamber (BEBC) built at CERN. It was with Gargamelle that a new form of weak interaction, that of the so-called neutral current, was discovered in 1973.

With CERN, Europe once more played a leading role in the development of high energy physics; and at CERN Bernard Gregory's exceptional organising abilities achieved rapid and widespread recognition on the European scene. Coming first to the laboratory as the leader of a French experimental team, he was appointed Directorate Member for Research in 1964, and then Director-General of CERN, succeeding Professor Victor Weisskopf on 1 January 1966. His five years as Director-General were very fruitful for CERN and European physics.

They were years which saw major developments at CERN, notably the construction of the Intersecting Storage Rings (ISR) and of the big bubble chambers already mentioned. But there were difficulties too. With the great variety of phenomena to be studied, Europe needed a new proton-synchrotron of much higher energy than the PS — a project that was to materialise later as the 400 GeV SPS (super proton-synchrotron), inaugurated in 1977. Its success, acclaimed today, was the outcome of the patient and delicate negotiations which marked the years of Gregory's leadership and in which he played a decisive part together with Edoardo Amaldi and John Adams. Europe owes him a great debt for the success of the SPS, which has become its main research tool in particle physics.

But Bernard Gregory was not only a research scientist and a great scientific administrator: he was also an outstanding teacher, whose contacts with pupils were direct and fertile. Having taught physics at the Ecole des Mines in Paris from 1951 to 1958, he took up a Professorship at the Ecole Polytechnique in 1959, where he was a prime mover in the revision of the physics course. When he left CERN in 1971, he returned to teaching at the Ecole Polytechnique, succeeding Leprince-Ringuet as head of the physics laboratory.

His successful achievements at European level brought him to a leading role in the French scientific community. In 1973 he was appointed head of the Centre National de la Recherche Scientifique, the main funding agency for scientific research in France, of which he had been a directorate member from 1963 to 1971. Though he was not long in this post, his influence was strong. Fundamental research was his great love, and he defended it vigorously through years of financial difficulties. He was the architect of better links between basic research and applied research, strengthening their interface (the so-called transfer sciences), and he developed interdisciplinary research, in particular on new sources of energy.

In 1976 he was appointed Délégué

Général à la Recherche Scientifique et Technique. Here at very high governmental level, as earlier at CERN and the CNRS, he was the obvious choice as the man best suited to shoulder the vast responsibilities involved in the organisation of research in France. But his ties with the European scientific community remained very close: at CERN above all, where he was Chairman of the ISR Committee from 1971 to 1973, French Delegate to the CERN Council from 1971, Vice-President of the Council in 1976, and finally its President-elect, looking forward with intense interest to the start of these new duties on 1 January 1978; but also at the European Science Foundation at Strasbourg and in numerous international negotiations in the scientific field.

Well known also outside Europe, he was Chairman of the Commission for Particles and Fields of the International Union of Pure and Applied Physics and, from August 1977, first Chairman of the International Committee for Future Accelerators (ICFA), a body bringing together for the first time representatives of all the regions of the world active in the field of big particle accelerators — the United States, Western Europe, the Eastern European countries and Japan.

Bernard Gregory's work and influence were of lasting value. His was a personality in which intellectual power and dignity were combined with warmth and simplicity. His passing represents a great loss for France and for Europe.

M. Jacob

L. Van Hove

P. A. Sheppard

PROFESSOR P. A. SHEPPARD, CBE, FRS, the meteorologist, died on 22 October 1977 in his 71st year after some years of failing health. He was respected equally in the academic world and in the political world of scientific affairs.

He left Bristol University in 1929 as a physics graduate to start work at Kew Observatory preparing for the International Polar Year 1932–33 which he was to spend as a member of a small British expedition working in the Northwest Territories of Canada. This led Sheppard to some original ideas on atmospheric electricity and ionisation near the ground but his life-long specialist interest began a little later when in 1934 he joined a team famous in meteorological history. This was set up by the War Office at Porton to study chemical warfare and led to extensive work on wind, temperature and humidity near the ground and especially their turbulent fluctuations.

In 1939 Sheppard joined Sir David Brunt in the Department of Meteorology at Imperial College and in 1952 succeeded him as professor, a post he held until becoming emeritus professor on retirement in 1974. During Sheppard's time the group at the Imperial College earned a world reputation as by far the most active and comprehensive university meteorological department in Britain—or the whole British Commonwealth for that matter—engaged mostly in research and post-graduate teaching.

Sheppard's specialist researches continued without break after the war and included an expedition in 1953 to Anegada, 18°N in the Virgin Islands, where a small group under his leadership made some unique observations of the conditions over the ocean up to some 1½ km height. Precision measure-

ments over the waters of Lough Neagh were also obtained, a technically difficult operation spread over a number of years.

In a complex field attracting attention in a growing number of countries including of course the United States and the Soviet Union it is not easy to assign any particular theoretical advance unequivocally to one person but Sheppard was certainly acknowledged as a leading thinker. His forte lay in always stressing physical processes and accurate measurements in a subject much favoured by mathematicians but he did as much as anyone to clarify the applicability to the atmosphere near the ground of the logarithmic variation of wind with height and the associated von Karman's non-dimensional constant with value 0.4.

As a general meteorologist Sheppard was much interested in the basic importance of his boundary layer studies for the largest scale problems of weather systems and the general circulation—the basis of world climate—and in this context showed that the convenient assumption of a turbulent boundary layer of limited thickness (of order 1 km) was not a valid approximation when applied to the drag of the wind on the surface. Neither, he thought, was the square law of wind drag in all circumstances, especially over the sea. His talent for lucid analysis and physical interpretation was highly regarded, as exemplified by his Presidential Address of 1958 to the Royal Meteorological Society — a triumph of exposition.

As time went on Sheppard took on wider responsibilities and from 1965–71 was chairman of the Space Policy and Grants Committee of the Science Research Council, most of the time a member of the SRC, and chairman also of the Scientific and Technical Com-

mittee of the European Space Research Organisation. These were onerous duties which occupied more of his energies than he had anticipated but he remained a meteorologist primarily and was continuously a member and much of the time chairman of the Meteorological Research Committee which guides the Meteorological Office in its researches. In this capacity he was intimately involved in the whole story of the revolution in weather forecasting from the mainly empirical methods which lasted through the 1940s to the elaborate computer calculations of the present day using mathematical models of growing sophistication derived entirely from the basic physics and incorporating Sheppard's specialities. Although so far as I know he never made a weather forecast in his life, in the way of duty, and for many years seemed to be working in a corner of the science remote from the main business of meteorology, it was gratifying to see his ideas incorporated in the scientific prediction models of today.

In addition to his CBE of 1963 and his FRS of 1964 Sheppard's honours included an honorary doctorate of Leningrad University, a rare tribute, and Hon. D.Sc. of Bath, the city of his schooldays.

Peter Sheppard, to use the familiar adopted name, had wide cultural and intellectual interests. He was forthright in his opinions and severe in scientific criticism but tempered by a warm and engaging personality. With his wife Phyllis who died in 1975—for him a tragic loss—he shared a love of travel and the countryside, of hospitality, good living and bright conversation. He will long be remembered with affection throughout his profession in many countries of the world and by a wider circle of friends. He leaves two successful sons.

R. C. Sutcliffe